A Proteomic Approach to Identify Biomarkers of 
Growth Hormone and Aging

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This dissertation titled

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ABSTRACT

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A Proteomic Approach to Identify Biomarkers of Growth Hormone and Aging (312 pp.)

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The growth hormone (GH)/ insulin-like growth factor-1 (IGF-1) axis is an important regulator of longitudinal growth, metabolism and aging. The physiological role of GH is manifested in mouse models with altered GH signaling. For example, bovine (b) GH transgenic mice are giant, lean, insulin resistant and short-lived. In contrast, GH receptor (R) gene knockout (GHR-/-) mice are dwarf, obese, insulin sensitive and long-lived. In order to uncover the biomarkers of GH and aging, two-dimensional gel electrophoresis (2-DE) has been used to analyze the mouse plasma proteome. The bGH, GHR-/- mice and their respective controls at different ages were analyzed. Female GHR-/- and control mice were also analyzed for gender differences.

Several biomarkers of aging and GH actions have been discovered. Biomarkers for aging include proteins whose plasma levels increased (isoform(s) of transthyretin (TTR), haptoglobin (Hp), immunoglobulin kappa chain) and decreased (isoform(s) of albumin, serum amyloid A-1 (SAA-1) and peroxiredoxin-2). Biomarkers for chronic GH action include upregulation of apolipoprotein (apo) E, Hp, mannose-binding protein-C, specific isoform(s) of alpha-2 macroglobin (a2m), retinol-binding protein-4 (RBP-4), and downregulation of apoA4, TTR, specific isoforms of apoA1, a2m and RBP-4. Importantly, isoforms of Hp and clusterin increased markedly in bGH mice during aging compared to wild type (WT) mice, possibly indicating an accelerated aging phenotype of...
these mice. Gender-specific biomarkers include plasma proteins that increased such as isoform(s) of apoE and RBP-4 or decreased such as isoform(s) of clusterin, albumin, Hp and hemoglobin-β in females. Interestingly, some proteins showed no change during male aging but significant increase (isoform(s) of apoA1, apoA4, TTR and albumin) or decrease (isoform(s) of RBP-4 and albumin) during female aging. In addition, several proteins (Hp, apoE and RBP-4) showed an interaction between genotype (GHR-/- and WT) and gender, suggesting interplay between GH and sex.

Finally, short-term effects of GH/IGF-1 were studied in mice injected with GH and/or IGF-1. GH injection resulted in upregulation of isoform(s) of apoE, Hp, RBP-4, SAA-1, albumin and a2m; and downregulated isoform(s) of apoA4 and clusterin. For IGF-1, isoform(s) of Hp, albumin and TTR were upregulated. Further, biomarkers differentiating between GH and IGF-1 actions have been revealed, including isoform(s) of apoE and TTR. The biomarkers of GH/IGF-1 injection will provide potential candidates for detecting GH and IGF-1 doping.

Approved: _____________________________________________________________

John J. Kopchick
Goll-Ohio Professor of Molecular Biology
This work is dedicated to my mother, Shi Hongxia, who passed away in the summer 2005 when I was in the USA pursuing my Ph.D. degree, a Pacific Ocean away. I wish I could have been there with you in those last few days. Losing you has been the hardest thing in my life. Sorry, mother, for not being there for you, while you had been there for me for 23 years. May you rest in peace!
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LIST OF ABBREVIATIONS

2-DE: two-dimensional electrophoresis
A2m: α-2 macroglobulin
Alb: albumin
ALS: acid-labile subunit
apoA1: apolipoprotein A1
apoA2: apolipoprotein A2
apoA4: apolipoprotein A4
apoE: apolipoprotein E
bGH: bovine growth hormone
Clu: clusterin
DAG: diacylglyceride
DTT: dithiothreitol
ELISA: enzyme-linked immunosorbent assay
FFA: free fatty acid
GH: growth hormone
GHR-/-: growth hormone receptor null or knockout
GHR: growth hormone receptor
GHRH: GH releasing hormone
HDL: high-density lipoprotein
ICTP: type I collagen telopeptide
IEF: isoelectric focusing
lg kappa C: lg kappa constant region
lg kappa V-III: lg kappa variable region III
lg kappa: Immunoglobulin kappa chain
IGF-1: insulin-like growth factor-1
IGFBP: IGF-1 binding protein
IL: interleukin
IPG: immobilized pH gradient
IR: insulin receptor
IRS: insulin receptor substrate
JAK: Janus kinase
JNK: c-Jun N-terminal kinase
LC: liquid chromatography
LDL: low density lipoprotein
MALDI: matrix assisted laser desorption ionization
MAPK: mitogen-activated protein kinase
MBP-C: mannose-binding protein-C
MS/MS: tandem mass spectrometry
MS: mass spectrometry
MT: metallothionein
Mw: molecular weight
PAGE: polyacrylamide gel electrophoresis
PEPCK: Phosphoenolpyruvate carboxykinase
PGC-1: PPAR-γ coactivator-1
pI: isoelectric focusing point
PI3K: phosphoinositide-3 kinase
P-III-P: procollagen type III
P-I-P: C-terminal propeptide of type I procollagen
PKC: protein kinase C
PPAR-γ: peroxisome proliferator-activated receptor-γ
Prx-2: peroxiredoxin-2
PTM: post-translational modification
RBP-4: retinol-binding protein-4
rhGH: recombinant human growth hormone
SAA-1: serum amyloid A-1
SDS: sodium dedecyle sulfate
SELDI: surface-enhanced laser desorption ionization
STAT: signal transducer and activator of transcription
TFA: trifluoroacetic acid
TG: triglyceride
TNF: tumor necrosis factor
TOF: time of flight
TTR: Transthyretin
UCP: uncoupling protein
VLDL: very low-density lipoprotein
WT: wild type
CHAPTER I. BACKGROUND

Review of GH and Mouse Models with Altered GH Signaling

*GH/IGF-1 axis*

Growth hormone (GH), a 191 amino acid (22-kDa) peptide hormone secreted by somatotrophs of the anterior pituitary gland, is regulated by growth hormone releasing hormone (GHRH) and somatostatin (*Figure 1*), both produced in the hypothalamus, and ghrelin which is produced in the stomach as well as several other tissues (Kopchick & Andry, 2000; G. S. Tannenbaum, 1991; A. O. Wong *et al.*, 2006). GH secretion is induced by GHRH, which is stimulated by stress, sleep and exercise (Garlaschi *et al.*, 1975; Reinhard W. Holl *et al.*, 1991; Kopchick, 2003; Krulich *et al.*, 1974; Manson & Wilmore, 1986; Osterman *et al.*, 1974; Rice *et al.*, 1975; Sutton & Lazarus, 1976). One protein induced by GH in tissues such as liver, muscle, fat and bone is insulin-like growth factor-1 (IGF-1), which mediates many of GH’s growth-promoting activities via endocrine, paracrine and/or autocrine routes. Thus, GH possesses both direct and indirect effects via IGF-1. These two physiological effects by GH have been termed the ‘dual effector system’ of GH action (Green *et al.*, 1985).

Both GH and IGF-1 can exert control of GH synthesis and secretion. Elevated blood IGF-1 levels will inhibit GHRH and GH release. This regulatory arrangement has been termed the long-loop feedback system. GH also negatively inhibits its own secretion and this regulatory system is called the short-loop feedback scheme (*Figure 1*).
GH plays important physiological roles in longitudinal growth and lactation as well as in the regulation of lipids, carbohydrates, and protein metabolism (Kopchick & Andry, 2000). GH also has a potent anabolic effect on bone and muscle and is lipolytic and anti hypogenic on adipose tissue (Cheek & Hill, 1974; Fryburg & Barrett, 1993; Katznelson, 2008; N. Moller & Jorgensen, 2009; Velloso, 2008; Widdowson et al.; Woodhouse et al., 2006).
GH exerts its potent action by signaling through GH receptors (GHRs) located on cell plasma membranes. GHR belongs to class I cytokine receptor superfamily and is a single pass transmembrane protein. Upon binding of GH to pre-dimerized GHR via two binding sites (W. Y. Chen et al., 1991; Cunningham et al., 1991), the two receptors rotate relative to each other (Brown et al., 2005), which leads to activation of intracellular Janus-kinase 2 (JAK2) (Frank et al., 1994), a kinase that phosphorylates itself as well as the GHR on multiple tyrosine (Y) residues Y332, Y487, Y534, Y566, and Y627 (L. H. Hansen et al., 1996; X. Wang et al., 1996). JAK2 further activates Signal Transducers and Activators of Transcription (STATs), which, upon tyrosyl phosphorylation, dimerize and enter the nucleus to regulate the transcription of GH-specific genes, among which is IGF-1 (Kopchick & Andry, 2000; Lanning & Carter-Su, 2006; Waters et al., 2006). Other major signaling pathways downstream of GHR include SHC-MAPK, IRS-PI3K and PKC (Figure 2) (Argetsinger & Carter-Su, 1996; Herrington & Carter-Su, 2001; Kopchick et al., 1999; Lanning & Carter-Su, 2006; Lichanska & Waters, 2008).
IGF-1, primarily induced by GH, is thought to mediate some of GH’s growth-promoting actions (Figure 1). About 75% of circulating IGF-1 comes from liver (Yakar et al., 1999), a primary GH target tissue. Other tissues such as muscle, fat and bone also express IGF-1, which acts via paracrine/autocrine routes. The paracrine/autocrine IGF-1 action is sufficient in mediating growth, because a liver-specific IGF-1 knockout (LID) mouse has normal growth and development activities, despite a 75% reduction in circulating IGF-1 (Yakar et al., 1999). In addition, GH and IGF-1 have their own specific effects. For example, GH can stimulate growth further in rats that have been stimulated with IGF-1 maximally (Fielder et al., 1996), and injection of GH and IGF-1 produce synergistic effects on bone growth (Ohlsson et al., 1998). GH stimulates the differentiation of precursor bone cells directly and induces IGF-1, which promotes the clonal expansion of the differentiated cells subsequently (Isaksson et al., 1982). Thus, the
growth promoting effect of GH is mediated in part by IGF-1, but GH has IGF-1 independent actions as well, further demonstrating the dual effects of GH action.

The GH/IGF-1 axis is involved in aging, as attenuated IGF-1 signaling over long term leads to increased lifespan in a wide range of organisms including yeast, worms, fruit flies, rodents and primates (Bonafè & Olivieri, 2009; Brown-Borg, 2009; Holzenberger et al., 2003; Tatar et al., 2003). Several mouse models with diminished GH/IGF-1 axis have extended lifespan (Longo & Finch, 2003). Ames and Snell dwarf mice have disrupted development of somatotrophs, lactotrophs and thyrotrophs in the pituitary due to mutations of Prop-1 and Pit-1, respectively. Although possessing a deficiency in GH as well as prolactin and thyroid stimulating hormone, these mice have increased lifespan, primarily due to decreased GH/IGF-1 action (A. Bartke, 2001; Wolf et al., 1993). The GHR-/- mouse also possesses an increased lifespan; in fact, it is the longest-lived laboratory mouse (Prize, 2009). The phenotypic characteristic of this mouse will be discussed below. Caloric restriction (CR) has been shown to increase lifespan in many species. Although the mechanism is still unclear, it is thought to be overlapping with an attenuated GH-IGF-1 axis (Bonkowski et al., 2009; Bonkowski et al., 2006; R. A. Miller et al., 2002).

In humans, elevated levels of GH in prepubertal children result in acromegalic gigantism (Ayuk & Sheppard, 2006). Excess GH secretion in adults leads to acromegaly, featured with enlarged bones (of the hands, feet, and head) and swelling soft tissues due to GH’s fluid retention actions. Complications of acromegaly include hypertension, diabetes, sleep apnea, kidney and heart problems (Chanson & Salenave, 2008). On the
other hand, GH deficiency (D) in children, if left untreated, results in dwarfism (Ayuk & Sheppard, 2006). GHD also occurs in adults, with symptoms of increased fat mass, reduced muscle size and strength and insulin resistance (Ho, 2007). GH insensitivity due to GHR mutations results in Laron syndrome (LS) (Laron, 1984) characterized by short stature, central obesity, as well as reduced muscle mass, force and exercise capacity (Ben-Dov et al., 2003; Brat et al., 1997; Laron et al., 2006).

Mouse models with altered GH/IGF-1 axis have been developed to study the action of GH/IGF-1. Bovine (b) GH transgenic mice, reminiscent of human acromegaly, have elevated IGF-1 levels in blood, are gigantic, lean, insulin resistant and die prematurely due to kidney lesions, liver tumors (Quaife et al., 1989) and perhaps heart dysfunction (Bollano et al., 2000; Izzard et al., 2009). On the other hand, GHR-/- mice mimic LS humans (Kopchick & Laron, 1999; Laron, 1984), and have very low blood levels of IGF-1, are dwarf, obese, insulin sensitive and long-lived (Berryman et al., 2004; Coschigano et al., 2003). Figure 3 shows the image of a giant bGH, a wild type (WT) and a dwarf GHR-/- mouse.

Figure 3. Image of an adult bGH, wild type (WT) and GHR-/- mouse.
Characteristics of GH transgenic mice

GH transgenic mice have high plasma insulin levels, with normal or low glucose (Table 1), suggesting these animals are insulin-resistant. Interestingly, they show normal or better glucose tolerance (A. Bartke et al., 2004; Olsson et al., 2005), at least when young, perhaps due to a robust pancreas (Parsons et al., 1995) that secretes increased insulin in response to a glucose challenge (Olsson et al., 2005). Peripheral tissues such as liver, muscle and fat all have shown insulin resistance (del Rincon et al., 2007; Fernando P. Dominici et al., 1999a; F. P. Dominici et al., 1999b), confirming the insulin-resistant state in these giant mice. Interestingly, a porcine GHR transgenic mouse model also shows insulin resistance in adipose tissue (pooled from three dopots: inguinal, parametrial and retroperitoneal) of 9-12 week-old mice (X. L. Chen et al., 2001), demonstrating an anti-insulin effect of increased GH signaling.

GH transgenic mice are lean and have altered lipid profiles compared to control mice (Table 2). They have decreased plasma free fatty acids (FFA), triglycerides (TG) and increased cholesterol including high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol. Liver cholesterol content also increases, but TG decreases. In addition, TG decreases in kidney, remains the same in muscle and increases in white adipose tissue (WAT) (del Rincon et al., 2007; Fernando P. Dominici et al., 1999a; F. P. Dominici et al., 1999b). Liver expression of genes involved in β-oxidation and lipogenesis are also reduced (Olsson et al., 2003). Plasma adiponectin, an insulin sensitizer, decreases, whereas resistin, TNF-α and IL-6 increase in GH mice (Zhihui Wang et al., 2007), which may contribute to insulin resistance of these mice.
Table 1 Comparison of insulin sensitivity in bGH versus WT control mice

<table>
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<tr>
<th>Genetic background and transgene used</th>
<th>Age</th>
<th>Gender</th>
<th>Glucose a</th>
<th>Insulin a</th>
<th>Glucose tolerance</th>
<th>Tissue insulin sensitivity b</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J PEPCK-bGH (Valera et al., 1993)</td>
<td>Not specified</td>
<td>Not specified</td>
<td>↔</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J PEPCK-bGH (Fernando P. Dominici et al., 1999a; F. P. Dominici et al., 1999b)</td>
<td>3-5 mo</td>
<td>Not specified</td>
<td>↔</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Frick et al., 2001a)</td>
<td>8 mo</td>
<td>Both</td>
<td>↓ (non-fasted)</td>
<td>↑ (non-fasted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Olsson et al., 2003)</td>
<td>6 mo</td>
<td>Male</td>
<td>↔</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J PEPCK-hGH mice (A. Bartke et al., 2004)</td>
<td>3 mo</td>
<td>Male</td>
<td>↓ (ns)</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Olsson et al., 2005)</td>
<td>5-6 mo</td>
<td>Male</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT- bGH (Berryman et al., 2006)</td>
<td>5 mo</td>
<td>Male</td>
<td>↓</td>
<td>↑ (ns)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J PEPCK-bGH (Zhihui Wang et al., 2007)</td>
<td>7 mo</td>
<td>Male</td>
<td>↔</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT- bGH (del Rincon et al., 2007)</td>
<td>3 mo</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>WAT ↓</td>
</tr>
</tbody>
</table>

a fasting blood glucose and insulin unless otherwise indicated
b tissue insulin sensitivity as measured by selected key insulin signaling molecules
Abbreviations: WAT: white adipose tissue, mo: month; ns: non-significant; ↑ increased as compared to WT mice, ↓ decreased, ↔ no change.
Table 2. Lipid profiles of GH versus WT control mice

<table>
<thead>
<tr>
<th>Genetic background and transgene used</th>
<th>Age</th>
<th>Gender</th>
<th>Plasma FFA a</th>
<th>Plasma TG a</th>
<th>Plasma cholesterol a</th>
<th>Liver cholesterol</th>
<th>Tissue TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J MT-bGH (Frick et al., 2001a)</td>
<td>8 mo</td>
<td>Both</td>
<td>↓</td>
<td>↓</td>
<td>↑ apoB ↔ LDL ↑</td>
<td>HDL ↑/ ↔</td>
<td>↑ Secretion ↓</td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Olsson et al., 2003)</td>
<td>6 mo</td>
<td>Male</td>
<td>↓ fed ↔ fasted</td>
<td>↑</td>
<td>↑ free↑ esterified ↓</td>
<td>Liver ↓</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J PEPCK-hGH mice (A. Bartke et al., 2004)</td>
<td>3 mo</td>
<td>Male</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Olsson et al., 2005)</td>
<td>5-6 mo</td>
<td>Male</td>
<td>↓ ns</td>
<td>↑ apoB ↔</td>
<td>↑</td>
<td>Liver Secretion ↔ Clearance ↓</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J PEPCK-bGH (Zhihui Wang et al., 2007)</td>
<td>7 mo</td>
<td>Male</td>
<td>↔</td>
<td>↓</td>
<td>↑</td>
<td>Muscle ↔ WAT ↑</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J MT-bGH (Palmer et al., 2009)</td>
<td>1 year</td>
<td>Both</td>
<td></td>
<td></td>
<td></td>
<td>Liver ↓ Kidney ↓</td>
<td></td>
</tr>
</tbody>
</table>

Fasting levels unless otherwise indicated
↑ increased as compared to control mice, ↓ decreased, ↔ no change.

The bGH mice have an increased lean mass and reduced fat mass compared to WT mice (Berryman et al., 2004; Palmer et al., 2009). They are also more resistant to diet-induced obesity (Berryman et al., 2006; Olsson et al., 2005). Interestingly, bGH mice have more absolute fat mass than WT mice before 6 months and when normalized to body weight, they still have more fat mass% than WT before 4 months of age (Palmer et al., 2009), suggesting an initial role of GH in adipocyte differentiation and proliferation. Moreover, GH has recently been shown to have depot-specific effects on adipose tissue. Preadipocytes isolated from subcutaneous region of GHR-/- mice are able to normally differentiate and proliferate, whereas adipocytes from parametrial fat pads are unable to do so (Flint et al., 2006), suggesting that GH is required for adipose tissue development.
in the parametrial but not subcutaneous depot. However, adult bGH mice have proportionally less subcutaneous fat mass than other depots compared to WT mice (Berryman et al., 2006), suggesting a more pronounced lipolytic action of GH in subcutaneous fat. Consistent with this finding is that GHR-/- mice have increased fat mass, with a large proportion in the subcutaneous region (Berryman et al., 2004; Berryman et al., 2006), indicating that a lack of GH action primarily affects subcutaneous fat. Thus, subcutaneous fat seems to be the most GH sensitive among all fat pads. Whether this is due to increased GHR remains to be determined and is currently under study (Miles, et al, in preparation).

Regarding energy intake and expenditure, 6-month old male bGH mice have similar normalized food and calorie intake (per body weight), increased oxygen consumption, increased liver uncoupling protein-2 (UCP-2) expression and 0.5 °C higher body temperature versus controls (Berryman et al., 2006; Olsson et al., 2005). However, unchanged body temperature, reduced resting metabolic rate and reduced cold-induced thermogenesis in GH versus control mice are also reported (Moura et al., 1998). The discrepancy is likely due to different experimental conditions, for example, the length of the measurement time and free moving versus constriction of the mice.

GH mice live on average for ~ 14 months, significantly shorter than WT littermates, which live for ~ 2 years (Berryman et al., 2004; McGrane et al., 1988; Steger et al., 1993). The pathophysiology of this animal model has been studied. Acromegalic patients often have kidney problems (Flyvbjerg, 1990; O'Shea & Layish, 1992). Likewise, GH transgenic mice have enlarged kidney (normalized with body weight) and develop
progressive glomerulosclerosis (T. Doi et al., 1990; T. Doi et al., 1988; Quaife et al., 1989), which features over-production of kidney mesangial extracellular matrix (T. Doi et al., 1991), increased inducible nitric oxide synthase expression in mesangial cells (S. Q. Doi et al., 2000), and glomerular podocyte damage (Reddy et al., 2007; Wanke et al., 2001). Together, these changes result in glomerulosclerosis and ultimately death of the animal.

Another abnormal organ in these mice is the liver. GH mice have disproportionally enlarged liver with large hepatocytes and develop liver tumors at old ages (Orian et al., 1989; Quaife et al., 1989; Shea et al., 1987), featuring increased rates of hepatocellular proliferation (Snibson et al., 2001; Snibson et al., 1999), sustained liver inflammation and hepatocellular injury with reduced natural killer T cells (Hardy et al., 1997). In line with this, GH mice have enlarged spleen and show altered immunity, including deficiency in T helper 2 cytokine production (Gonzalo et al., 1996) and self-antibody production leading to arthritic disorders (Ogueta et al., 2000). Reduced capacity to fight oxidative stress may also be a cause of premature aging. It was found that female hGH mice had early declines of antioxidant enzyme superoxide dismutase (SOD) in the kidney and reduced levels of glutathione peroxidase (GPx) in the liver, increasing the risk of ROS damage (Steven J. Hauck & Bartke, 2001).

One complication of acromegaly is hypertension and increased cardiovascular disease risks (Colao et al., 2001). The cardiovascular physiology of GH mice has been studied. The bGH mice have enlarged hearts even after normalization to body size (Bohlooly et al., 2001a; Dilley & Schwartz, 1989; Izzard et al., 2009). Female bGH mice are reported to have normal blood pressure, a normal vascular wall mass and cross-
sectional areas of vessels in proportion to body size, normal organization and branching patterns of vessels, but increased wall-to-lumen ratio of mesenteric vasculature (Dilley & Schwartz, 1989). The latter feature is similar to that seen in hypertensive vascular remodeling. Normal blood pressure but impaired cardiac function including suppressed systolic function and impaired energy reserve are reported in 6-8 month old female bGH mice (Bollano et al., 2000). However, cardiac contractile function is enhanced in isolated ventricular myocytes from GH mice; therefore, the impaired cardiac function is unlikely due to dysfunctional cardiocytes (Colligan et al., 2002). However, cardiocyte apoptosis is affected in GH mice. Chronic exposure to GH affects cardiac apoptosis in an age-dependent manner such that young (3 mo) male bGH mice have reduced apoptosis whereas older (9 mo) ones have increased apoptosis versus control mice (Bogazzi et al., 2008). Increased cardiac apoptosis may contribute to the impaired cardiac function of GH mice at older ages. GH mice also have endothelial dysfunction in carotid artery caused by increased oxidative stress, which is more pronounced in aged (6 mo) versus young (2.5 mo) female mice (I. J. Andersson et al., 2006a). Metabolically, heart is also affected by chronic GH actions. Compared to age-matched controls, young male bGH hearts have increased but older bGH mice have decreased fatty acid oxidation (Bogazzi et al., 2009).

Others report increased blood pressure but with normal vasculature functions in male and female bGH mice 5-8 mo of age (Bohlooly et al., 2001a; Izzard et al., 2009). Consistent with this, GHR-/- mice have reduced blood pressure (E. Egcioglu et al., 2007; Izzard et al., 2009), plasma rennin and aortic eNOS expression (E. Egcioglu et al., 2007). The renin-angiotensin-aldosterone-system plays a central role in hypertension. GH mice of both genders have increased aldosterone levels independent of rennin, potentially contributing to hypertension observed in some studies involving this mouse model.
The controversy regarding blood pressure and cardiac functions of GH mice is not clear, but different ages used in different studies may be a factor.

Since GH affects connective tissue, male bGH mice have thicker skin, but this effect seems to be dependent on sex hormones, since male bGH mice have normalized skin when castrated (Wanke et al., 1999). Male hGH mice have thicker skin than controls with increased dermis, whereas female hGH mice have thinner skin than controls due to a reduced hypodermis (Serrat et al., 2007). These studies demonstrate another sexually dimorphic effect of GH and its interaction with sex hormones related to skin thickness.

Several other abnormalities of bGH mice have been noted. BGH mice have normal lymphatic vessels in normal skin, but increased lymphatic vessels ingrowth into the granulation tissue of full-thickness skin wounds (Banziger-Tobler et al., 2008). They display more spontaneous locomotor activity (Bohlooly et al., 2001b; Soderpalm et al., 1999). They have altered metabolism of neurotransmitters in the brain (A. Bartke et al., 2002). BGH mice display reduced sympathetic responsiveness as well as plasma and tissue noradrenaline concentrations in 2-6 mo old females (I. J. Andersson et al., 2004).

In summary, chronic GH excess promotes a giant and lean body phenotype. The organisms are hyperinsulinemic with normal or low glucose levels, and a relatively short lifespan. The accelerated aging seen in GH transgenic mice involves abnormal pathology of multiple organs, with altered metabolism and increased oxidative stress, leading to liver pathology and tumors, kidney damage, and cardiac dysfunction. One note is that many of these adverse phenotypes are observed when animals get older rather than young, suggesting it is important to study them in an extended age span rather than only at young ages. The gender difference also calls for more research in both genders rather than merely males.
**Characteristics of GHR-/- mice**

GHR-/- mice have been shown repeatedly to be insulin sensitive (Table 3). GHR-/- mice have lower glucose levels, although they tend to normalize at older ages. They possess lower insulin levels throughout lifespan compared to WT mice. Glucose tolerance, however, is impaired, perhaps due to a hypo-pancreas phenotype (Liu et al., 2004; Robertson et al., 2006) that results in reduced insulin secretion relative to WT mice upon glucose stimulus (Guo et al., 2005). Regarding tissue insulin signaling, the GHR-/- liver has similar insulin sensitivity compared to control mice; however, the GHR-/- heart and muscle are more sensitive to insulin (Table 3).

GHR-/- mice are obese and accumulate excess subcutaneous fat (Berryman et al., 2004; Berryman et al., 2006; Liu et al., 2004). However, they have decreased plasma TG and cholesterol as well as liver cholesterol (Table 4). Plasma FFA, liver TG as well as muscle cholesterol and TG are all similar compared to controls. GHR-/- mice have lower body temperature (S. J. Hauck et al., 2001), increased energy intake (normalized to body weight) when young (2-4 mo) but similar compared to controls when older (9 mo) (Berryman et al., 2006; Coschigano et al., 2003; Emil Egecioglu et al., 2006). GHR-/- mice have increased oxygen consumption (Berryman et al., 2006; Westbrook et al., 2009) with a lower respiratory quotient (RQ) values indicative of fat oxidation (Westbrook et al., 2009).
Table 3. Comparison of insulin sensitivity parameters in GHR-/- versus WT control mice

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Age</th>
<th>Gender</th>
<th>Glucose a</th>
<th>Insulin a</th>
<th>Glucose tolerance b</th>
<th>Tissue insulin sensitivity c</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J (F. P. Dominici et al., 2000)</td>
<td>3-5 mo</td>
<td>Not specified</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td>Liver ↔</td>
</tr>
<tr>
<td>C57BL/6J (S. J. Hauck et al., 2001)</td>
<td>5 mo</td>
<td>Both</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>129/Ola-BALB/c and C57BL/6J (Coschigano et al., 2003)</td>
<td>1.5, 2, 3, 5, 7, 9, 11 mo</td>
<td>Male</td>
<td>1.5-9 mo ↓, 11 mo ↔</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>129/Ola-BALB/c-C57BL/6J (Liu et al., 2004)</td>
<td>3, 10, 60, 180 days</td>
<td>Both</td>
<td>↓</td>
<td>↓</td>
<td>Insulin tolerance ↑</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (A. Bartke et al., 2004)</td>
<td>3 mo</td>
<td>Male</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (Guo et al., 2005)</td>
<td>2-4 mo</td>
<td>Both</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (Al-Regaiey et al., 2005)</td>
<td>21 mo</td>
<td>Male</td>
<td>↓ (ns)</td>
<td>↓</td>
<td></td>
<td>Liver ↓</td>
</tr>
<tr>
<td>C57BL/6J (Masternak et al., 2005b)</td>
<td>21 mo</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Liver ↑ Muscle ↔</td>
</tr>
<tr>
<td>129/Ola-BALB/c (E. Egecioglu et al., 2005)</td>
<td>3.5 mo</td>
<td>Male</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (Berryman et al., 2006)</td>
<td>5 mo</td>
<td>Male</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (Masternak et al., 2006)</td>
<td>3 mo 21 mo</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Heart ↑ 3mo ↔ 21 mo</td>
</tr>
<tr>
<td>129/Ola-Balb/c-C57BL/6J (Robertson et al., 2006)</td>
<td>6 mo</td>
<td>Not specified</td>
<td></td>
<td></td>
<td></td>
<td>Liver ↔ Muscle ↔ but delayed signaling</td>
</tr>
<tr>
<td>C57BL/6J (Al-Regaiey et al., 2007)</td>
<td>21 mo</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Muscle ↑</td>
</tr>
<tr>
<td>C57BL/6J (Giani et al., 2008)</td>
<td>14 mo</td>
<td>No specified</td>
<td></td>
<td></td>
<td></td>
<td>Heart ↑</td>
</tr>
<tr>
<td>C57BL/6J (Bonkowski et al., 2009)</td>
<td>12 mo</td>
<td>Male</td>
<td>↔</td>
<td>↓</td>
<td></td>
<td>Liver ↔ Muscle ↑</td>
</tr>
</tbody>
</table>

a. fasting blood glucose and insulin  
b. sometimes insulin tolerance as indicated  
c. tissue insulin sensitivity as measured by selected key insulin signaling molecules  
↑ increased as compared to control mice, ↓ decreased, ↔ no change.
Table 4. Lipid profiles of GHR-/- versus WT control mice

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Age</th>
<th>Gender</th>
<th>Plasma FFA a</th>
<th>Plasma TG a</th>
<th>Plasma cholesterol a</th>
<th>Liver cholesterol</th>
<th>Tissue TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J (A. Bartke et al., 2004)</td>
<td>3 mo</td>
<td>Male</td>
<td>↔</td>
<td>↓</td>
<td>free↓ esterified↑</td>
<td>Liver ↔</td>
<td></td>
</tr>
<tr>
<td>129/Ola-BALB/c-C57BL/6J (Liu et al., 2004)</td>
<td>3-4 mo</td>
<td>Both</td>
<td>↓ ns</td>
<td>↓ ns</td>
<td>HDL↓</td>
<td>Muscle ↔</td>
<td></td>
</tr>
<tr>
<td>129/Ola-BALB/c (E. Egecioglu et al., 2005)</td>
<td>3.5 mo</td>
<td>Male</td>
<td>↓ apoB↓</td>
<td>↓ HDL↓</td>
<td>LDL↓</td>
<td>Muscle ↔</td>
<td></td>
</tr>
<tr>
<td>C57BL/6J (Masternak et al., 2005a)</td>
<td>21 mo</td>
<td>Male</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>Muscle ↔</td>
<td></td>
</tr>
</tbody>
</table>

a. Fasting levels
↑ increased as compared to control mice, ↓ decreased, ↔ no change.

GHR-/- mice are long-lived, and the mechanism is thought to be similar to that of caloric restriction (CR), since CR and GHR disruption have a partial overlap in terms of gene expression (R. A. Miller et al., 2002). Also, CR does not further improve the lifespan of GHR-/- mice (Bonkowski et al., 2009).

In contrast to GH transgenic mice which have progressive glomerulosclerosis, GHR-/- mice are protected from streptozotocin-induced kidney damage (Bellush et al., 2000). However, GHR-/- mice do not have increased antioxidant enzymes or free radical scavenge in liver and kidney compared to controls (S. J. Hauck et al., 2002), suggesting at least in these two tissues, oxidative stress is not the mechanism to explain the protection from aging.

GHR-/- mice have altered cardiac parameters but maintain a normal function. They have reduced heart weight and volume (E. Egecioglu et al., 2007; Izzard et al., 2009), but manage to produce normal cardiac output to meet their oxygen requirement (E. Egecioglu et al., 2007). They have slightly reduced heart beat rate, reduced blood pressure, but
normal contractile response to norepinephrine and acetylcholine (E. Egecioglu et al., 2007; Izzard et al., 2009).

As with LS individuals (Shevah & Laron, 2007), GHR-/- mice have decreased cancer incidence, including breast, lung, pituitary, lymph and prostate (Ikeno et al., 2009; Kineman et al., 2001; Z. Wang et al., 2005; X. Zhang et al., 2007).

In summary, GHR-/- mice are obese, have altered lipid profiles, yet have increased insulin sensitivity and longevity. Together with GH transgenic mice, they make excellent animal models to study GH actions. Many issues remain unresolved such as the mechanism of longevity with altered GH signaling. Genomic and proteomic tools provide approaches that are now being used in attempt to add to the knowledge base of molecular mechanisms responsible for GH’s physiological effects. My proteomic studies hopefully will contribute to this field of study.
Overview of Plasma Proteome and Biomarkers

*Plasma proteome*

Blood is composed of blood cells and plasma. In general, blood cells include red blood cells or erythrocytes, white blood cells or leukocytes and platelets or thrombocytes. Serum is the cell-free, clear liquid of blood devoid of clotting factors, whereas plasma contains the coagulation factors, and is usually obtained by treating blood with anticoagulants such as heparin. Other than clotting factors, plasma and serum contain the same proteins. For simplicity, ‘plasma’ is used throughout this dissertation unless when serum, not plasma, is involved.

Plasma proteins can be classified into several groups (N. Leigh Anderson & Anderson, 2002). Classical or typical plasma proteins are the resident proteins that carry out specific functions in plasma. These are secreted primarily by liver and intestine and are usually larger than 45 kDa and are, thus, not filtered by the kidney. Examples include albumin, apolipoproteins, transferrins and transthyretin. Plasma proteins smaller than 45 kDa often form multimers or bind to other proteins/lipids/polysaccharides in order to escape kidney filtration. Immunoglobulins are a second group of plasma proteins. Hormones and cytokines are a third group of plasma proteins with varying molecular masses and half-lives. These are usually present in relatively small concentrations (ng/ml or less). Unusually high levels of hormones may cause deleterious effects. For example, excess GH causes acromegaly. The last group includes tissue leakage products, proteins released into blood stream due to cell death or damage, as well as aberrant secretions released from tumors or diseased tissues. Examples include cardiac troponins, creatine
kinase, and myoglobin that are used in the diagnosis of myocardial infarction (N. Leigh Anderson & Anderson, 2002).

The term proteome refers to the entire set of proteins expressed in a certain cell, tissue, or organism at a specific time under a defined condition (N. L. Anderson & Anderson, 1998; James, 1997). The plasma proteome is the entire protein content present in plasma. The plasma proteome contains subsets of other tissue proteomes, and is, therefore, the most complex proteome (N. Leigh Anderson & Anderson, 2002). As expected, many of the identified plasma proteins are cellular proteins (presumably released due to cell rupture); however unexpectedly, many examples of nuclear, cytoplasmic, and kinesin complex proteins have been identified (N. Leigh Anderson et al., 2004). In the mouse proteome, it is found that a large proportion of the identified proteins are membrane proteins, the majority of which are presumably shed extracellular domains of membrane proteins and receptors (Hood et al., 2005).

One major challenge in studying plasma proteome is the dynamic range of proteins. The majority of serum proteome is composed of albumin, transferrins, immunoglobulins and apolipoproteins. In this regard, 22 proteins comprise 99% of the plasma proteome (Figure 4). The most and least abundant proteins differ by 10 orders of magnitude (Figure 5) (N. Leigh Anderson & Anderson, 2002). For example, albumin has a concentration of 35-50 g/L, whereas the concentration of GH in normal human plasma is 0-3 ng/L. The abundant proteins mask the less abundant ones making it difficult to study the latter.
**Figure 4.** Twenty-two proteins make up 99% of plasma protein mass. Reproduced from (N. Leigh Anderson & Anderson, 2002).

**Figure 5.** Plasma protein abundance spans 10 orders of magnitude. X axis shows representative protein names. Reproduced from (N. Leigh Anderson & Anderson, 2002).
Proteomic techniques to study plasma proteome

Traditional methods to study plasma proteins include chemical fractionation, biochemical assay of enzyme activity, targeted detection using polyclonal or monoclonal antibodies, as well as commercialized kits such as enzyme-linked immunosorbent assays (ELISAs). Recently, proteomic techniques, especially 2-DE, have been developed to study the proteome of various tissues including blood (Banks et al., 2000; Naaby-Hansen et al., 2001; Pandey & Mann, 2000; Rabilloud, 2000).

Since its introduction in 1975 (Klose, 1975; O'Farrell, 1975), 2-DE has been used to separate many proteins including plasma proteins. In the current human plasma proteome SWISS-2DPAGE database (Sanchez et al., 1995), there are 1966 proteins (spots on the gel) detected, 626 of which have been identified. Seventy entries are present in SWISS-PROT database (database, 2009) (the small # of plasma proteins in SWISS database may be due to the low rate of conversion from literature reports to database, as for an entry to appear in the database, stringent confirmation is needed). An annotated (protein spots with identification) 2-DE gel map for human plasma proteins is shown in Figure 6.
Figure 6. SWISS-2DPAGE map of human plasma proteins (database, 2009). X axis: isoelectric focusing point; y axis: molecular weight in kDa.

2-DE is only the isolating step. Following 2-DE and staining of the separated proteins, they are identified by mass spectrometry (MS), the gold standard of protein identification in modern proteomics research (Packer & Harrison, 1998). Figure 7 shows a typical flow chart of this process. Proteins are digested by an enzyme such as trypsin, chymotrypsin or pepsin, with trypsin the most commonly used, and the resultant fragments are resolved as a mass spectrum based on time of flight (TOF) of individual peptides through a MS analyzer. In MALDI-TOF, individual peptides are mixed with a solvent and a matrix then placed on a probe tip. Under a vacuum condition, the solvent
evaporizes, leaving the peptides dispersed evenly into the matrix, which then is charged by a pulsed laser beam. The charged peptides then go through an electromagnetic field and reach the detector after a TOF that reflects mass (m)/charge (z) specific to the peptides. Since all peptides have the same charge of 1+, the TOF corresponds to the mass of the peptides. Therefore, the spectrum conveys information on peptide mass distributions. The spectrum can then be compared to proteins in the database that have been digested with trypsin in-silico, and depending on the degree of similarity, the target protein can be matched to a known protein in the database. In MS/MS, each digested peptide is further ionized into sub-fragments, which undergo another round of TOF resulting in several spectra for each peptide. Because the peptides are ionized into random fragments, an MS/MS spectrum provides information that can be used to deduce the amino acid sequence of a given peptide. Together, MS and MS/MS provide more confident identification of a given protein. One common search engine used for peptide mass fingerprint (data derived from MS) and MS/MS ion search is MASCOT at Matrixscience (http://www.matrixscience.com/search_form_select.html).
Proteins are often subjected to post-translational modifications (PTMs), such as phosphorylation, glycosylation, acetylation, oxidation and methylation. There are 593 PTMs listed in Unimod database (http://www.unimod.org/modifications_list.php?). Some of the PTMs are in vivo or in vitro processes (i.e., phosphorylation) whereas others are derived from experimental conditions (i.e., carbamidomethyl of Cys by alkylation with iodoacetamide to prevent disulfide bond reformation in 2-DE). PTMs can be identified by MS and MS/MS by selecting fixed or variable PTMs. For example, if a protein is known to have ‘carbamidomethyl’ group on the Cys residue (as seen in proteins treated with iodoacetamide in 2-DE), then ‘carbamidomethyl’ can be selected for ‘fixed modification’ so that MASCOT will add that group on Cys residue of proteins in the database in-silico.
to compare with the actual MS and MS/MS data. On the other hand, if a protein is suspected to be phosphorylated, then ‘phosphorylation’ can be selected for ‘variable modification’; by doing this, the search engine will modify proteins in the database with possible phosphorylations in-silico and compare to the actual MS and MS/MS data for best matches.

The advantage of 2-DE coupled with MS and MS/MS is that 2-DE itself provides information on Mw and pI of intact proteins, which can serve as an internal confirmation for protein identification. Also, a given protein is unequivocally identified to a particular spot. Further, protein spots on the gel with suspected PTMs may be correlated with MS fingerprint and MS/MS spectra. Thus, 2-DE based proteomic approach has a relative high identification rate, about 50-70% (Conrotto & Souchelnytskyi, 2008). The disadvantage of 2-DE is low-throughput and that experienced experts are required for reproducible results (Conrotto & Souchelnytskyi, 2008).

Liquid chromatography (LC) is another proteomic technique. It is commonly used for high-throughput analysis of samples. In LC, a sample mixture is digested by a protease such as trypsin, and the mixed peptides are loaded onto a column and separated using hydrophobic phase chromatography. Peptides eluted from the column are then ionized and subsequently identified by MS/MS. There are quite a few derivatives of this technique, including reverse phase LC and ion exchange LC. The advantage of this technique is that automation has been developed such that the eluted peptides are directly subjected to MS ionization; thus, high-throughput, high reproducibility and high sensitivity are characteristics of this technique. The major disadvantage of LC is that
peptides, instead of intact proteins, are eluted and analyzed, therefore peptide information has to be merged to resolve the identity of the original protein; thus, relatively low protein identification rates are associated with this technique. Approximately 5-10% of the spectra can be identified, although this is to an extent compensated for by the large number of spectra generated (Conrotto & Souchelnytskyi, 2008).

Recently, a new technique based on protein binding called protein arrays or protein chips has been developed (MacBeath & Schreiber, 2000). Proteins that are known to bind specific proteins are placed on a chip, and the sample is then exposed to the chip, allowing specific binding to occur. Following washing, fluorescence or radio isotope intensities are determined. Limitations with this technique include difficulty in protein quantification and the ability to generate binding antibodies (Conrotto & Souchelnytskyi, 2008). Also, significant knowledge about protein-protein interaction and binding affinity is required for the protein bound to the chip and the protein that will recognize it.

With the advancement in technology, the list of identified plasma proteins is growing. In 2002, about 300 proteins were known (N. Leigh Anderson & Anderson, 2002). Since then, using ultra-high-efficiency strong cation exchange LC/reversed-phase LC/MS/MS, 800 proteins have been identified in human plasma (Shen et al., 2004). Compiling from all previous reports, a non-redundant list of 1175 distinct gene products is generated for human plasma proteome (N. Leigh Anderson et al., 2004). Subsequently, 81 more proteins have been identified by multidimensional (multiple rounds of separation based on protein charges and hydrophobicity) LC separation (Sheng et al., 2006), adding to the growing list and results in a total of 1256 identified proteins in human plasma.
Plasma proteins as biomarkers

Because blood is easy to obtain and the procedure is less invasive compared to tissue biopsies, it is used routinely for disease diagnostics. Plasma proteins have long been used as biomarkers for diseases. For example, cardiac troponins T (Gerhardt & Ljungdahl, 1998), creatine phosphokinase (Michelson, 1980) and lactate dehydrogenase (Metais & Sacrez, 1963) in plasma are highly sensitive markers of heart injury. Similarly, liver transaminases (Molander et al., 1955) as well as interleukin-18 levels (Vecchiet et al., 2005) are closely associated with liver injury. Additionally, plasma biomarkers are used for clinical diagnosis for various diseases including cancer (Bates, 1991), inflammation (Ridker et al., 2000), diabetes (Dworacka & Winiarska, 2005), multiple sclerosis (Angelucci et al., 2005), tuberculosis (Djoba Siawaya et al., 2009), HIV infection (Mine et al., 2009; Rose et al., 2009) and infections by various pathogens.

Plasma proteins can serve as biomarkers not only for diseases, but also for any specified condition, state or process. For example, this project was started with the hope of identifying biomarkers for aging. Also, plasma proteins that change as a function of hormonal action have been determined. For example, serum IGF-1 levels increase in response to GH. Thus, IGF-1 is a plasma indicator of GH activity (Kemp et al., 1981; D. C. Moore et al., 1982; Reiter & Lovinger, 1981).

Similar to research in determining the plasma proteome, proteomic techniques including 2-DE have been used to discover novel biomarkers of diseases (X. Huang et al., 2006; Villanueva et al., 2006; R. Zhang et al., 2004). For example, squamous cell carcinoma antigen 1 was found to be a biomarker of tongue cancer (X. Huang et al.,
Aiming to uncover specific proteins that show significantly different expression patterns from the entire protein set, proteomic techniques provide a promising approach for biomarker discovery. In order to aid the discovery and characterization of biomarkers, a Human Proteome Detection and Quantitation Project has been founded, attempting to establish splice variants, PTMs, protein-protein interactions, and tissue localization of a given protein via proteomic means (N. Leigh Anderson et al., 2009).

**Mouse plasma proteome**

The mouse and human share a high degree of genome similarity or homology (Carver & Stubbs, 1997) and have very similar chromosomal organization and content ("Initial sequencing and comparative analysis of the mouse genome", 2002). About 80% of mouse proteins have a strict 1:1 orthologue in the human genome ("Initial sequencing and comparative analysis of the mouse genome", 2002; Pennacchio, 2003). It is much easier to conduct biological research using the mouse, since genetic and environmental variations can be controlled. Therefore, the mouse is a good model organism for the study of human diseases.

The mouse plasma proteome is less well studied than human. As in human plasma, the majority of the mouse plasma proteome is composed of a few abundant protein species. In a recent study, 12,300 unique peptides that originated from 4567 unique proteins, about 16% of all known mouse genes (which is ~28,000), have been identified through multidimensional fractionation and MS/MS approach in mouse plasma (Figure 8) (Hood et al., 2005). Thus much more research remains to be performed in the mouse plasma proteomic field.
As has been mentioned earlier, the SWISS-2DPAGE database includes 2D annotation of human plasma proteins, but to date, no reference gel exists in the database for the mouse plasma proteome. However, many reports have been published using 2-DE on mouse plasma or serum proteins to uncover biomarkers of diseases including lung adenocarcinoma, hepatocellular carcinoma, tongue cancer, injuries from burn or radiation, as well as biomarkers of therapeutics against snake venom and liver cirrhosis (Bijon & Jürgen, 2007, 2009; Duan et al., 2004; Gazzana & Borlak, 2008; Guipaud et al., 2007; Hao et al., 2002; Hsueh-Fen et al., 2004; X. Huang et al., 2006; W. Li et al., 2007; Ma et al., 2003; Ren et al., 2007; Roberto et al., 2008; Skehel et al., 2000; Yuichiro et al., 2006). Among these papers, a few have published reference 2D PAGE maps (Bijon & Jürgen, 2007, 2009; Duan et al., 2004; Gazzana & Borlak, 2008; Guipaud et al., 2007; Ren et al., 2007; Roberto et al., 2008; Skehel et al., 2000). Therefore, 2-DE has become an increasingly popular technique for biomarker discovery in mouse models.
Introduction to 2-DE

The proteomic technique 2-DE has been used to separate proteins for several decades. It was first introduced in 1975 (Klose, 1975; O'Farrell, 1975), and a few years later, in 1977, 2-DE was used to isolate human plasma proteins (L. Anderson & Anderson, 1977). In the first dimension, proteins with different isoelectric points (pIs) are focused at different positions in an immobilized pH gradient (IPG) strip, in which a series of immobilized reagents with a broad pI range are chemically linked to an acrylamide matrix strip. In the second dimension, proteins from the IPG strips are separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) based on size. With varying gel concentrations, it is possible to separate proteins in different molecular weight ranges. Following SDS-PAGE, the gels are stained to visualize the proteins, and the gel image is captured using software that determines the density of each protein spot. From these images, proteins in the gel are profiled and quantified. Proteins of interest are then removed from the gel and identified by MS and/or MS/MS.

Sample preparation

Proteins must be solubilized, denatured, and reduced before being absorbed onto IPG strips. Cells and/or tissues are usually lysed to release the protein content. Body fluids such as plasma, serum, urine, or cerebral spinal fluid do not need lysing.

For cultured cells, gentle lysis methods are sufficient to release cellular proteins (T. S. Berkelman, T, 1998). Osmotic lysis works well with blood cells and cultured cells, which are suspended in a hypotonic solution and burst because of osmotic pressure (Ramsby et al., 1994). Sonication is also a method employed to lyse cells. One cautionary
note is that prolonged sonication can overheat the sample. Therefore, samples are usually sonicated with short bursts and cooled on ice between bursts. Another method is freeze-thaw lysis (Toda et al., 1994). Cells are rapidly frozen using liquid nitrogen then thawed in one or more cycles. For tissues that are difficult to disrupt such as liver, kidney, and heart, a mortar and pestle can be used to grind the tissues frozen in liquid nitrogen (Gorg et al., 1988). Various mechanical homogenization devices are also available to efficiently lyse many types of tissues (Goldberg, 2008; Posch et al., 1995).

Cells and tissues are usually lysed in the presence of detergents to solubilize and denature proteins. Ionic detergents are avoided since they interfere with the subsequent IEF. If ionic detergents such as SDS are used, the sample must be diluted by excess non-ionic detergents or removed by acetone precipitation. Centrifugation is usually carried out afterwards to collect the protein supernatant, or differential centrifugation can be performed to recover proteins in various cellular organelles (Castle, 2003).

**Contaminant removal**

Often tissue or blood samples need to be further processed to remove contaminants such as salt, DNA, RNA, and polysaccharides that may interfere with the separation or visualization of proteins on a 2D gel. Excess salt and other small ionic molecules make the sample more conductive and should be kept at a minimum prior to IEF. Dialysis or precipitation can be used to desalt the sample. DNA and RNA can increase sample viscosity, and may result in background protein smearing or ‘clogged’ gel pores. DNase/RNase is used to hydrolyze the nucleic acid polymers in the sample. Large polysaccharides also can block gel pores and cause horizontal streaking. They are
removed by precipitation with ammonium sulfate or phenol/ammonium acetate. Lipids can reduce the solubility and change the pI and/or molecular weight (Mw) of proteins. They can be removed by excess detergents or acetone precipitation (T. Berkelman, 2008). This is especially important when analyzing adipose tissue samples.

**Protein quantification, solubilization/denaturation/reduction/alkylation**

After the preliminary sample treatment, the protein content of the sample must be quantified to determine the volume and mass to be used for 2-DE. Standard protein assays, such as the Bradford assay, are used to determine protein concentration in a sample. In Bradford assay, a protein specimen, along with a known gradient concentration of a standard protein such as bovine serum albumin, reacts with a Rosaniline dye, and the protein concentration in the specimen is determined by colorimetry (Bradford, 1976). The proper amount of protein needed for 2-DE (for example, 500ug) is calculated and diluted in a buffer containing urea, thiourea, non-ionic and/or zwitterionic detergents, a reducing agent, carrier ampholytes and protease inhibitors (Kopchick *et al.*, 2002). Urea is a neutral chaotrope that denatures proteins (Rabilloud & Chevallet, 2000). Heating aids the solubilization but should be avoided because with heat urea will break down into ammonium cyanate, which can cause protein charge modifications (Cejka *et al.*, 1968). Thiourea is often used in addition to urea to improve solubilization and denaturation. Non-ionic detergents such as NP-40 and Triton X-100, and/or zwitterionic detergents such as CHAPS, are included to further solubilize proteins and prevent hydrophobic aggregation. Carrier ampholytes are added to create a pH gradient during IEF. A reducing agent such as dithiothreitol (DTT), beta-
mercaptoethanol, or tributylphosphine (TBP) is added to reduce disulfide bonds (Kopchick et al., 2002). Following reduction, the sulfhydryl groups on the cysteine residues should be alkylated by an agent, such as iodoacetamide, to prevent sulfide bond reformation (Herbert et al., 2001). During sample preparation, protease inhibitor cocktails are usually added at the time of solubilization/denaturation/reduction, and are often added as early as cell/tissue lysis (T. S. Berkelman, T, 1998).

**Electrophoresis**

After sample preparation, proteins are first separated by IEF. Two methods exist for creating a pH gradient. One uses carrier ampholytes to generate pH gradients in polyacrylamide tube gels. A more widely-used method is the use of IPG strips. The strips are generated by covalently linking acidic and basic buffering groups to a polyacrylamide gel strip. This eliminates the ‘drifting’ which can occur in the carrier ampholyte-based method. IPG strips are commercially available in various pH ranges with linear or non-linear pH gradients that amplify a specific pI range (T. S. Berkelman, T, 1998).

After IEF, proteins in the strip are subjected to the second dimension SDS-PAGE (Laemmli, 1970). In this procedure, the proteins are coated with SDS in proportion to their mass (Shapiro et al., 1967), which makes them negatively charged. Because of the ‘pores’ in the polyacrylamide, larger proteins move more slowly and smaller proteins faster; thus, the separation is based on the mass of the proteins. Also, one can selectively separate proteins in a particular molecular mass range using specific concentration of acrylamide in the resolving gel. Larger proteins would be separated better in lower percentages of acrylamide and vice versa for smaller proteins.
Staining of protein spots

Following SDS–PAGE, proteins are stained using a variety of dyes including coomassie blue or a silver stain. Coomassie blue staining is simple and quantitative but relatively insensitive to small amount of proteins. Silver staining is sensitive but the linearity is not satisfactory. In addition, silver stain interferes with subsequent MS analysis (Lauber et al., 2001). Fluorescent stains such as sypro red, ruby, and orange have high sensitivity, an excellent linear range and does not affect subsequent MS analysis.

Plasma as a sample for 2-DE

Blood is the most common tissue collected for clinical diagnostics. Centrifugation at 7,000 x g for 10min effectively removes the blood cells and the supernatant plasma is readily soluble. The sample can be treated for 1st dimension IEF directly, since normally very low concentrations of DNA, RNA, large polysaccharides or lipids are present in blood. See Figure 9B for a typical 2D gel image of mouse plasma proteins.

As mentioned in the previous section, a few abundant proteins dominate in plasma, obscuring the presence of other less abundant proteins. Only 1% of plasma proteome, albeit in low abundance, contains a large amount of unique proteins and therefore represents a biomarker reservoir. In a typical 2D gel of plasma proteins, albumin will appear as a huge blot masking many other proteins (Figure 9A). Therefore, it is necessary to deplete albumin and other high-abundance proteins in order to resolve the remaining proteins. Human albumin can be depleted with immunoaffinity resin (Steel et al., 2003),
resulting in greatly improved resolution of the 2D image (Echan et al., 2005). However, albumin depletion often causes loss of other proteins that bind to it (Granger et al., 2005).

Figure 9. 2D gel images of plasma proteins. A, 2D gel image of plasma proteins with albumin smear. Adapted from (Ding et al., 2009). B, 2D gel image of mouse plasma proteins. Enclosed in the box are smaller proteins that are not affected by albumin smear.

Recently a new technique using a hexapeptide ligand library has been developed (Guerrier et al., 2008). Plasma proteins are bound by their respective hexapeptide ligands; the more abundant a protein is, the more likely it will be bound, resulting in enriched population of less abundant proteins. This technique minimizes the loss of proteins as can occur in immunodepletion, but requires a large sample volume (~1 ml plasma). This is not a problem in human samples, but for animal studies such as mice or rats, it is difficult to obtain such a large volume per animal.

A method used to circumvent the albumin smear as well as the pitfalls of albumin-depletion is simply to selectively study only proteins with a relative small molecular weight. Albumin’s molecular mass is ~70 kDa, the majority of which consists of a protein smear above 50 kDa on a 2-D gel. By increasing the 2nd dimension gel
concentration to 15%, proteins larger than 45 kDa are clustered as horizontal streaks in the upper region of the gel (Figure 9B). When focusing only on proteins smaller than 45 kDa, the albumin smear is not encountered (Figure 9B).

This method has limitation on the size range of proteins measured, but provides a faster, simpler way to study plasma protein profile. There are already a handful of proteins in the low molecular weight (LMW) range that have been identified, and studies have shown that LMW proteins below 30 kDa contain comprehensive categories of proteins including structural proteins, nuclear proteins, transcription factors, channels, receptors, enzymes, secreted proteins, coagulation and complement factors, transport and binding proteins, proteases, protease inhibitors, homones and cytokines (Figure 10) (Tirumalai et al., 2003) and have great potential for biomarker discovery (Petricoin et al., 2002).

Figure 10. Relative numbers of proteins identified in LMW proteome. Hypothetical proteins are proteins that have not been experimentally identified but whose sequences are predicted from known open reading frames. Reproduced from (Tirumalai et al., 2003).
Recombinant human GH doping in sports

Because GH promotes bone, cartilage and muscle growth as well as fat degradation, athletes often abuse recombinant human GH (rhGH) to enhance performance. RhGH is on the list of prohibited substances by the World Anti Doping Agency (WADA) (WADA, 2009). Since GH was first extracted from human pituitary, it has been used in clinical practice to treat children with GH deficiency (D) (Figure 11). RhGH has been approved by the FDA to treat children with the following conditions: Prader-Willi Syndrome, children born small for gestational age, Turner’s Syndrome, Noonan’s Syndrome, chronic renal failure, idiopathic short stature and cystic fibrosis (Krysiak et al., 2007); in adults, rhGH has been approved to treat adult GHD (Cummings & Merriam, 1999), wasting syndrome of AIDS (Mulligan et al., 1993; Schambelan et al., 1996) and short bowel syndrome (Krysiak et al., 2007). However, with the beneficial effects of GH come the abuse and misuse of GH among athletes. Although officially on the prohibited list as early as 1989, it continues being abused and more and more cases and scandals have been exposed (Figure 11). It is estimated that the dose of rhGH abuse is 10-25 IU/day (Saugy et al., 2006), which is much higher than therapeutic doses for adult GHD, which is 0.2-0.3mg/day (~0.6-0.9 IU/day) (Ho, 2007). Moreover, rhGH is thought to be used in combination with other doping agents such as anabolic steroids or erythropoietin (Saugy et al., 2006).
It is difficult to detect GH doping. First, rhGH is identical to the endogenous hGH. Thus, it is difficult to directly detect ‘doped’ GH levels in the blood. Second, endogenous hGH is secreted in a pulsatile manner and the levels are affected by many environmental factors such as exercise, sleep, stress and nutritional status (Garlaschi et al., 1975; Reinhard W. Holl et al., 1991; Kopchick, 2003; Krulich et al., 1974; Manson & Wilmore, 1986; Osterman et al., 1974; Rice et al., 1975; Sutton & Lazarus, 1976). Third, GH has a very short plasma half life of about 15min (R. W. Holl et al., 1993). Exogenous GH is cleared quickly from circulation (Owens et al., 1973). Thus, ‘the window of opportunity’ for detection of rhGH is only 24-36h (Wu et al., 1999). Urine samples, often used to test drug doping among athletes, is not feasible because urine GH levels are very low (Albini...
et al., 1988) and poorly correlated with serum GH levels (Flanagan et al., 1997). To date, blood is the most common biological fluid used for GH doping detection.

Current approaches to detect GH doping

To date, there are two approaches to detect rhGH doping in blood. One is based on the different isoforms of hGH. Endogenous GH, produced in the somatotrophs of the pituitary gland, has several forms including the most abundant 22 kDa isoform, a 20 kDa isoform (Baumann) generated by alternative precursor RNA splicing (Leung et al., 2002), and other minor-isoforms with sizes of 17.08 kDa and 17.84 kDa. These latter isoforms are detected by proteomic analysis in the human pituitary gland but not plasma (Zhan et al., 2005), although the degraded GH isoforms might be due to a sample extraction artifact (Baumann). Also, hGH is subjected to PTMs including acetylation, deamidation, phosphorylation and possibly glycosylation (Baumann; Zhan et al., 2005). It is well known that IGF-1 is induced by GH, resulting in a feedback inhibition of endogenous hGH secretion. Since rhGH is composed of only the 22 kDa isoform, a change in the ratio of 22 kDa isoform over the total GH makes it possible to detect whether exogenous rhGH has been used (Bidlingmaier et al., 2000; Wu et al., 1999).

The limitation of this approach is that the window of opportunity for detection is short (Nelson & Ho, 2008) and it is impractical to draw blood from athletes daily. The GH isoform method was adopted by WADA for the 2004 Athens and 2006 Turin Olympic Games. However, no positive results were found possibly due to the above reasoning.
A second approach to detect GH doping is to determine GH-dependent biomarkers with longer half lives than GH. Two groups of proteins are currently used for this purpose. One is IGF-1/IGF binding proteins (IGFBPs) and the other includes proteins involved in bone and collagen turnover. A study entitled ‘The GH-2000 project’ was initiated to search for GH specific biomarkers in human serum (Dall et al., 2000; Holt et al., 2009b; Longobardi et al., 2000; Sonksen). In a randomized, double blind, placebo-controlled study involving healthy volunteers of both sexes, daily rhGH was administrated (0.1 IU/kg/day and 0.2 IU/kg/day) for 4 weeks followed by a 8-week wash-out period and resulted in increases in IGF-1, acid-labile subunit (ALS) and IGFBP-3 levels (Dall et al., 2000). Markers of bone and collagen turnover were also increased. These included osteocalcin, a C-terminal propeptide of type I procollagen (P-I-P), a bone resorption marker type I collagen telopeptide (ICTP), and a soft tissue marker procollagen type III (P-III-P). Of these markers, only P-III-P and osteocalcin remained significantly elevated after the 8-week wash-out period (Longobardi et al., 2000). IGF-1 and P-III-P are considered ideal candidate markers because of little variation from day-to-day, or by exercise and gender (Sonksen). In ‘the GH-2000 project’, females were found to be less responsive than males to GH treatment as has been reported by others (Nelson et al., 2008). This is presumably due to the antagonistic actions of estrogen on GH action (Meinhardt & Ho, 2007). Although females are less responsive, the GH-2000 project has established IGF-1 and P-III-P (Figure 12) as two biomarkers of GH that may be used to detect GH doping.
Figure 12. Individual datapoint plot of P-III-P against IGF-I levels in male subjects after 3 weeks of treatment. Reproduced from (Sonksen, 2009).

Injury is quite common among athletes and it can cause changes in serum bone and collagen markers. Concerns about injury and ethnicity differences on GH doping markers resulted in a new phase of study: the GH-2004 project (Holt et al., 2009a; Holt et al., 2009b). Elite and amateur athletes have been followed longitudinally for sport-related injury. IGF-1 does not change after injury. A raise in P-III-P serum level is found; however, this rise is minimal compared to GH’s effect (Erotokritou-Mulligan et al., 2008a). Ethnicity reveals some differences in IGF-1 and P-III-P, but still within 99% limit of confidence (Erotokritou-Mulligan et al., 2008b; Erotokritou-Mulligan et al., 2009). Similarly, another study found that ethnicity is a moderate effector on GH-responsive markers (Nelson et al., 2006).

Another independent double blind, placebo controlled rhGH injection study, the Kreischa study, confirmed IGF-1 and P-III-P as GH-responsive markers (Kniess et al., 2003). These two studies have developed respective algorithm to calculate a score to
predict the likelihood of GH doping. The GH-2000 formula \[ \text{GH-2000 test score} = -2.269 + 0.7207 \log (P-III-P) + 0.5210 \log (IGF-1) \] is able to detect 90% of those receiving rhGH in the Kreischa study with a similar sensitivity. The Kreischa formula \[ \text{Kreischa test score} = -13.465 + 0.0272 \times IGF-I + 0.0398 \times IGFBP-3 - 1.367 P-III-P - 0.00271 \times (IGF-I P-III-P) \] can correctly identify 41% of individuals receiving GH in the GH-2000 study (Erotokritou-Mulligan et al., 2007).

**Novel biomarkers for GH are needed**

GH is affected by many factors such as exercise, gender, age, body mass index (BMI), etc. The effect of maximum exercise on endogenous hGH levels has been examined in elite athletes (Ehrnborg et al., 2003). HGH shows a peak concentration by the end of the exercise period and decreases to baseline levels within 30-60 min. Similarly, IGF-I, IGFBP-3, ALS and IGFBP-2 increase following exercise, with IGF-I, IGFBP-3 and ALS returning to baseline by 3-4 days. The bone markers ICTP and P-III-P increase after exercise but return to normal within 120 min whereas osteocalcin and P-I-P are not affected by exercise (Ehrnborg et al., 2003). This study demonstrates that although exercise increases the levels of several biomarkers, the effect is acute and diminishes in a relatively short period of time (J. D. Wallace et al., 1999; J. D. Wallace et al., 2000).

Demographic factors and sport activity or category have been examined for their effects on GH responsive biomarkers (Nelson et al., 2006). Age is the major contributor to variability in these marker values that significantly decrease with age. Gender significantly confounds the variability of IGFBP-3 and ALS, whereas BMI and sport
category are only modest effectors. Taken together, these results point out that reference ranges of biomarkers must be established for different factors including age and gender.

In addition, the candidate biomarkers IGF-1 and P-III-P have relatively large variations among individuals or even within the same person. For example, serum samples obtained over a 2- to 3-week period from 1103 elite athletes have shown that the within-subject coefficient of variation (CV, a value to measure the variance relative to the mean) is nearly 21% for the IGF axis markers and 15% for the collagen markers (Nguyen et al., 2008). In addition, in some cases, IGF-1 levels cannot properly reflect that of GH. For example, some acromegalic patients have high levels of IGF-1 but GH levels are normal; thus, the correlation of GH and IGF-1 is poor. Also, after treatment to normalize GH, IGF-1 does not always normalize (Barkan, 2007; Clemmons, 2006; Popovic, 2005; Stonesifer et al., 1981). Thus, other plasma biomarkers for GH action would be valuable. It is generally agreed that a single biomarker is not a robust indicator of GH doping; rather, a combination of multiple biomarkers will generate more robust and reliable results.

Theoretically, the whole plasma proteome could be profiled before and after rhGH treatment and those that are differentially regulated could be identified. Thus, proteomics represents a novel and highly promising approach to discover additional biomarkers for rhGH doping. Surface plasmon resonance (SPR) immune assays (Gutiérrez-Gallego et al., 2009) and surface-enhanced laser desorption ionization (SELDI)–TOF MS (Chung & Baxter, 2009) are two emerging proteomic techniques to uncover new GH specific markers. SPR is the phenomenon which occurs when light is reflected off thin metal
films. With this technique, a thin metal film is immersed in a medium. A change in the light reflective angle ($\theta$) and the light intensity can be observed when a difference in the medium occurs such as when GH binds to its antibody. SPR immune assays thus can differentiate between different GH isoforms which produce different $\theta$ and/or light intensities when binding to an antibody (Gutiérrez-Gallego et al., 2009).

SELDI-TOF MS is similar to MALDI-TOF MS introduced earlier in the section. Like in MALDI, digested peptides are mixed with the matrix, but in SELDI, the mixture is applied to a surface that binds to the peptides with different affinities, instead of subjected to TOF directly as in MALDI. Thus, SELDI has one dimension of peptide separation before TOF. Recently, hemoglobin $\alpha$-chain has been discovered to be a biomarker of GH action using this SELDI-TOF MS technique (Chung et al., 2006), although the robustness of this marker is brought into question because subsequent analysis find this marker to be at very low levels or even undetectable, possibly due to a different material of the tubes used for sample collection (Chung & Baxter, 2009). To date, a validated novel biomarker of GH is yet to be discovered. One project of this Ph.D. dissertation involves using a classic proteomic tool, 2-DE, to discover biomarkers for GH.
CHAPTER II. RESEARCH OBJECTIVES

My research interest is to discover novel biomarkers of GH as it relates to aging. These markers will not only serve as indicators of GH and aging, but also provide insight into the mechanism(s) of aging as well as the in vivo interaction between GH and aging. Since GH is involved in the aging process, it would be interesting to study GH action in the context of aging. Mouse models with altered GH signaling, specifically, bGH and GHR-/- mice were used in my research. My specific research objectives are to discover the biomarkers of aging and GH action.

Longitudinal studies involving bGH transgenic mice and littermate controls were carried out to identify biomarkers of aging in the context of constant and elevated levels of GH action. Longitudinal studies of GHR-/- mice and littermate controls (male and female) were carried out to identify biomarkers of aging in the context of the lack of GH action. Blood from these mice were collected periodically and plasma samples gathered for 2-DE analysis. Plasma proteins that exhibited significant differences during aging, between different genotypes as well as gender differences were identified. To complement the longitudinal studies, a cross-sectional study involving WT male mice was designed to confirm the biomarkers of aging found in the longitudinal study.

My studies of GH transgenic and GHR-/- mice involved mice in which the transgene was constitutively present or absent. I have therefore extended my studies of the plasma proteome to mice injected with either GH or IGF-1. The goal was to discover biomarkers of short-term action of GH and IGF-1. This study will hopefully determine GH and IGF-1 responsive proteins that could then be evaluated in human GH doping.
studies. Ultimately, it may result in the development of new targets for GH doping detection.

I will organize my results in four papers in the following four chapters (chapters III to VI). The first one (chapter III) is centered on aging biomarkers in WT male mice, both in a longitudinal and a cross-sectional setting. The first paper will also include the 2D map and identification of all plasma proteins pertinent to this dissertation. The first paper also describes in detail the key techniques and methods such as 2-DE and MS which are referred to by subsequent papers. The second paper (chapter IV) involves plasma proteins found in male bGH compared to WT mice during different ages. The third one (chapter V) focuses on GHR-/- and WT mice of both genders at different ages. The final manuscript (chapter VI) is the short-term GH/IGF-1 injection of WT male mice. Each of these papers is independent; however, each subsequent paper also relates to the previous ones. The four papers are summarized in Table 5.
Table 5. Organization of four papers in this dissertation

<table>
<thead>
<tr>
<th></th>
<th>Paper 1 Chapter III</th>
<th>Paper 2 Chapter IV</th>
<th>Paper 3 Chapter V</th>
<th>Paper 4 Chapter VI</th>
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</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td>WT (C57BL/6J)</td>
<td>WT and bGH (C57BL/6J)</td>
<td>WT and GHR-/- (C57BL/6J)</td>
<td>WT (C57BL/6J)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
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<td>Male</td>
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<td>Male</td>
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<tr>
<td><strong>Ages</strong></td>
<td>Longitudinal: 2, 4, 8, 12, 16, 19 months Cross-sectional: 6, 12, 24 months</td>
<td>Longitudinal: 2, 4, 8, 12, 16, 19 months</td>
<td>Longitudinal: 8, 16, 24 months</td>
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</tr>
<tr>
<td><strong>Objective</strong></td>
<td>Biomarkers of aging</td>
<td>Biomarkers of chronic GH action; aging pattern of bGH versus WT mice</td>
<td>Biomarkers of lack of GH action; biomarkers of gender; interaction of gender and aging; interaction of gender and genotype</td>
<td>Biomarkers of short-term GH action (GH doping); biomarkers of IGF-1 doping; differential effects of GH and IGF-1</td>
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<tr>
<td><strong>Parameters measured</strong></td>
<td>Plasma proteomics</td>
<td>Body weight and body composition</td>
<td>Fasting glucose and insulin (except for paper 4)</td>
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</tbody>
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My hypothesis is as the following: 1) The levels of a subset of plasma proteins will change during aging. These proteins will be biomarkers of aging. 2) The levels of a subset of plasma proteins will be up or downregulated in bGH compared to WT mice. These proteins will be biomarkers of (chronic) GH action. 3) The levels of a subset of plasma proteins will be up or downregulated in GHR-/- compared to WT mice. These proteins will be biomarkers of lack of GH action. 4) A subset of plasma proteins will show a different trend of change during aging in bGH and/or GHR-/- mice compared to WT mice; these will be biomarkers of the interaction between aging and GH. 5) A subset of plasma proteins will show a difference between males and females. 6) A subset of
plasma proteins will be different in GH or IGF-1 injected mice versus controls. These proteins will represent biomarkers of short-term GH or IGF-1 action. 7) A subset of plasma proteins may be different between GH and IGF-1 treatments. These proteins will be biomarkers to differentiate between GH and IGF-1 actions.
CHAPTER III. BIOMARKERS OF AGING: PROTEOMIC ANALYSIS OF PLASMA FROM WILD TYPE MALE MICE OF DIFFERENT AGES

Abstract

To discover biomarkers for aging, we analyzed plasma proteins of normal male mice using two-dimensional electrophoresis coupled with mass spectrometry. This proteomic approach allowed us to identify 86 plasma proteins, 13 of which exhibited significant changes during aging. Interestingly, many of these proteins exist as protein isoforms with distinct masses and/or charges probably resulting from yet unknown post-translational modifications. The levels of several plasma proteins changed through aging. For example, several proteins increased significantly including one isoform of transthyretin, two isoforms of haptoglobin and three isoforms of immunoglobulin kappa chain. Conversely, several proteins decreased significantly during aging, including peroxiredoxin-2, serum amyloid A-1 and five isoforms of albumin. Thus, proteomic analysis of plasma proteins has identified several plasma markers of mouse aging.
Introduction

Aging is an inevitable process that eventually leads to death. Through medical intervention, it is now being debated whether there are viable therapeutic targets that would delay the onset of or slow the aging process. If possible, therapeutic agents may ultimately extend human life span, and perhaps more importantly, improve the quality of life or the healthspan of the elderly (Benedetti et al., 2008; Boudrault et al., 2008; Nass et al., 2009; Topinkova, 2008).

Biomarkers of aging are biological characteristics that indicate or represent certain age periods. For example, there are markers for puberty- Tanner stages of puberty (Marshall, 1986), adulthood, middle age and old age. Traditional biomarkers for old age include wrinkles, gray hair, reduced muscle strength, and increased susceptibility to infections. In a general term, old age is associated with frailty, a condition featured with disability and comorbidities (Nass et al., 2009; Topinkova, 2008). These macroscopic traits, however, are composed of complex physiological processes and are therefore difficult to study. Other aging biomarkers currently in use include evaluation of cardiorespiratory function (blood pressure, forced expiratory capacity, etc.), hemotology (red and white blood cells, hematocrit [percentage volume that red blood cells occupy in blood]), and serum biochemical markers including the levels of albumin, globulin and blood urea nitrogen (Nakamura & Miyao, 2007, 2008). It has been found that systolic blood pressure and blood urea nitrogen increase with chronological age, whereas forced expiratory volume in 1 second, hematocrit and albumin decrease (Nakamura & Miyao, 2007). Another example of a biomarker is dehydroepiandrosterone sulfate, an adrenal
steroid sulphate, which has been found to decline during normal aging in humans (Orentreich et al., 1992) and rhesus monkeys (Lane et al., 1997).

Serum/plasma proteins are relatively easy to access and are widely used for disease diagnosis. Serum/plasma biomarkers of aging will not only facilitate the elucidation of the physiology processes of aging, but also may provide insight in therapeutic targets and intervention strategies to delay and/or slow aging.

Proteomic techniques including two-dimensional electrophoresis (2-DE) have been used to study aging (Ballesteros et al., 2001; Gromov et al., 2003; Miura et al., 2007; Sato et al., 2006; Schöneich, 2003; Toda, 2000; Yuri Miura, 2005). This technique has led to the identification of many candidate proteins that are altered by aging. For example, Prx-2, an antioxidant enzyme, is able to be induced by irradiation in cultured astrocytes from young but less so from old rats; also phosphorylation of α-tubulin is increased with aging (Miura et al., 2007; Yuri Miura, 2005). Senescent bacteria cells produce aberrant isoforms of proteins that are oxidized (Ballesteros et al., 2001). Old human skin expresses altered levels of certain proteins, including manganese-superoxide dismutase, tryptophanyl-tRNA synthetase, the p85β subunit of phosphatidylinositol 3-kinase, and proteasomal proteins PA28-α (Gromov et al., 2003). N-glycosylated proteins are found to accumulate in aged rat cerebral cortex (Sato et al., 2006). Since proteomics reveals the total detectable proteins in a given sample, this approach will make it possible to identify proteins involved in the aging process. Here we use 2-DE as a tool to uncover biomarkers of aging in mouse plasma.
Materials and Methods

*Experimental animals*

For the longitudinal study, male C57 BL/6J mice (n=8) were followed through 2, 4, 8, 12, 16 and 19 months of age. These ages represent post-puberty (2 months), young adult (4 months), adult (8 months), middle-age (12 and 16 months) and old age (19 months) (Richard A. Miller & Nadon, 2000). For the cross-sectional study, three different groups of male C57 BL/6J mice of 6 months (n=6), 12 months (n=8) and 24 months (n=8) were used. These represent young adult, middle age and old age in mice, respectively (Richard A. Miller & Nadon, 2000). Mice were housed 2-3 per cage at room temperature (22 °C) in a 12-hour light, 12-hour dark cycle. All mice were fed ad lib with a typical chow diet. Animal protocols were approved by Ohio University’s Institutional Animal Care and Use Committee.

*Body composition measurement*

Body composition was measured using an NMR analyzer the Bruker Minispec (The Woodlands, TX) as described previously (List *et al.*, 2009; Palmer *et al.*, 2009). The data are presented as absolute mass (gram or g) of lean mass, fat mass and fluid mass. Each animal was measured twice and the mean value was reported.

*Plasma collection*

Blood was collected into heparinized capillary tubes following tail tip clipping after heat lamp exposure. Whole blood was centrifuged at 7000 x g for 10 minutes at 4 °C and the resulting plasma was stored at -80 °C.

*Fasting glucose and insulin measurements*

Mice used in the longitudinal study were fasted for 4 hours and bled at 3 PM. Blood glucose was measured by ONE TOUCH glucometer from Lifescan (Milpitis, CA).
Plasma insulin levels were determined using an ultrasensitive rat/mouse insulin ELISA kit (ALPCO, Windham, NH).

**2-DE**

2-DE was performed as previously described (Ding et al., 2009; Kopchick et al., 2002; List et al., 2007; Qiu et al., 2005; Sackmann-Sala et al., 2009). 2-DE was carried out within a week after plasma collection to avoid possible sample degradation.

Plasma proteins were treated for 2 hours at room temperature with sample buffer containing 8M urea, 1.8M thiourea, 4% zwitterionic detergent (CHAPS), and 5mM reducing agent tributylphosphine, in a protease inhibitor cocktail containing AEBSF, aprotinin, leupeptin, bestatin, pepstatin A and E-64 (Sigma-Aldrich, Inc., St. Louis, MO). Then 15mM iodoacetamide was added for alkylation for 30min. 500ug protein was loaded onto a 17cm immobile pH gradient gel (IPG) strip with a broad linear pI range of 3–10 (Bio-Rad, Hercules, CA). After actively rehydrated (50V) for 12 hours at 20 °C using a Protean IEF cell (Bio-Rad), the strips were subjected to first dimensional isoelectric focusing (IEF) at 4000V for 60000V-Hr. When IEF was completed, strips were incubated in buffer containing 2% (w/v) sodium dodecyl sulfate (SDS), 0.5M Tris/HCl (pH 6.8), 20% (v/v) glycerol for 25min. The middle section of the strip with pI 5-8 bearing the majority of plasma proteins was removed and subjected to second dimension SDS polyacrylamide gel electrophoresis (PAGE). Only the middle section of the strip was used instead of the full length because the middle section contained most of the proteins already and the full length strip required a large gel. Polyacrylamide (15%) gels (8 x 7cm) were used for the 2nd dimension electrophoresis at a current of 25mA/gel until a total of 250 V-Hr was reached. After electrophoresis, the gels were fixed in a solution containing 40% ethanol and 2% acetic acid overnight followed by washing three
times in 2% acetic acid. The gels were then stained with SYPRO Orange (1:5000) (Molecular Probes, Eugene, OR).

**Quantification of proteins**

Gel images were captured using a laser-scanner Pharos FX plus (Bio-Rad) with an excitation wavelength of 488nm and an emission wavelength of 604nm. Proteins were matched across all images using PDQuest (Bio-Rad) software and manually checked and corrected when necessary. For quantification, the intensity of each protein spot was determined according to the fluorescence signal strength, and then normalized by the total density of each image using PDQuest software. The data were exported to an excel sheet, log-transformed and then subjected to statistical analysis.

**Statistical analysis**

All statistical analyses were performed using SPSS version 14.0 software (Chicago, IL). For the longitudinal experiment, data were subjected to ‘repeated measures’ for significant effect of aging (p<0.05 for insulin or glucose and p<0.01 as a more stringent cutoff for protein intensity). For the cross-sectional experiment, data were subjected to one way ANOVA (p<0.01 for protein intensity). In the analysis of body composition, one way ANOVA was applied followed by LSD as a post hoc test (p<0.05). All data were presented as the mean ± SEM.

**Protein identification by mass spectrometry (MS), MS/MS, and liquid chromatography (LC)-MS/MS**

Proteins of interest were excised manually from the SDS-PAGE gels, lyophilized and shipped to Protea Biosciences, Inc. (Morgantown, WV) for MS and MS/MS analyses.
using matrix assisted-laser desorption ionization (MALDI)-time of flight (TOF) and MALDI-TOF-TOF. Selected proteins were also subjected to LC-MS/MS.

**Protein in-gel digestion (Performed at Protea Biosciences, Inc.)**

Gel plugs were treated with acetonitrile and 50mM ammonium bicarbonate, then reduced and alkylated with 250mM dithiothreitol (DTT) (60min / 55°C) and 650mM iodoacetamide (60min / room temperature / in the dark). Digestion was performed with 500ng trypsin in 50mM ammonium bicarbonate buffer overnight. Peptides were extracted using 5% formic acid in 50% acetonitrile (dehydration), followed by rehydration with 50mM ammonium bicarbonate. For each extraction step, the solution was aspirated, collected, and collated. Three extraction cycles (dehydration and rehydration) were performed per sample. The recovered peptides were lyophilized, reconstituted in 10mM acetic acid, and re-lyophilized to yield a purified, protein digest.

**MALDI target spotting (Performed at Protea Biosciences, Inc.)**

After in-gel digestion by trypsin, proteins were analyzed by ABI 4800 MALDI TOF-TOF analyzer. A C18 ProteaTip was washed and then equilibrated a using 0.1% trifluoroacetic acid (TFA) / 50% acetonitrile solution and a 0.1% TFA / 2% acetonitrile solution. The remaining reconstituted protein digest solution in an auto sampler vial (~65% of sample) was loaded onto the C18 ProteaTip by aspirating and expelling the sample 5-10 times within the sample vial. The bound sample was washed twice with the 0.1%TFA / 2% acetonitrile solution by aspirating and expelling 20µL of the wash solution 5-10 times. The sample was spotted directly onto a MALDI target that was pre-
spotted with 0.6µL MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) using 1µL of an elution solution (0.1%TFA / 90% acetonitrile).

The MALDI MS parameters used for analyses were: MS acquisition in reflector mode; positive ion mode; mass range = mass/charge (m/z) = 850 – 4000; 400 laser shots per spectrum; minimum signal/noise (S/N) = 10 for MS acquisition; 15 strongest precursors chosen for MS/MS; minimum S/N = 30 for MS/MS precursors; MALDI spot interrogated until at least 4 peaks in the MSMS spectra achieved a S/N = 70.

Protein identification from combined MS and MS/MS data (Performed at Protea Biosciences, Inc.)

The Applied Biosystems GPS Explorer™v3.6 program was used for MS/MS data processing with Mascot (Mascot, 2009) as the search engine. Other parameters included: sample type: gel samples; digestion enzyme: trypsin; species: mouse; database: NCBInr; type of search: combined MS and MS/MS; mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 1 Da; maximum missed cleavages: 2; variable modifications: oxidation (M), carbamidomethyl (C); exclusion mass list: 1151.8, 1358.9, 1795.1, 2211.4, 2225.4, 2283.

LC-MALDI spotting

Some protein spots that did not yield good identification by MS/MS were selected for LC- MALDI analysis by ABI Tempo LC MALDI using Tempo LC MALDI v.2.00.09 as a data acquisition and processing program. Lyophilized digested samples were reconstituted in 7µL of 0.1% TFA in deionized water and 5µL of sample was injected into a separation column (Merck Chromolith CapRod monolith column – 150 X 0.1mm)
with a separation gradient using 0.1% acetic acid 2% acetonitrile and 0.1 % acetic acid in 90% acetonitrile for 30 min.

**Protein identification from LC-MALDI MS/MS data**

The ABI Protein ProteinPilot Software 2.0 Program was used for MS/MS data processing with ABI’s MS/MS search engine software Paragon. Other parameters included sample type: *identification*; digestion enzyme: *trypsin*; special factors: *gel-based I.D.*; species: *mouse*; I.D Focus: *biological modifications*; database: *NCBI*.

**Manual confirmation of protein identification from MS and MS/MS data**

*(performed at Ohio University)*

MS and MS/MS data were manually submitted to MASCOT (Mascot, 2009) for confirmation of the results reported by Protea Biosciences. For MS data, the searching criteria were as follows: *NCBI* as the database; *mouse* as the species; *trypsin* digestion; *maximum one missed cleavage*; fixed *carbamidomethylation of Cys*; variable modifications of *oxidation-M (methionine)*, *pyro-Glu*; *monoisotopic*; and 50 ppm of peptide mass or parent tolerance. For MS/MS ion search, in addition to the above conditions, a peptide charge of +1 and a fragment mass tolerance of 0.5 Da were used.
Results

**Body composition, insulin and glucose levels during aging**

There were significant age differences in body weight, lean, fat and fluid masses (one-way ANOVA, p<0.01, cross-sectional setting). Body weight and lean mass became significantly higher in mice of 12 months compared to 6 months and did not change thereafter (Figure 13A,C). Fat mass increased from 6 months to 12, 19 and 24 months, but decreased from 19 to 24 months (Figure 13D). As a result, body weight increased as mice aged but slightly decreased after 19 months. Fluid mass became higher at 9 and 12 months and increased further at 19 and 24 months (Figure 13E). The changes in body composition can be seen in Figure 13B, in which lean and fluid masses increased, whereas fat mass increased till 19 months and then decreased at 24 months.

*Figure 13.* Body composition of WT mice at different ages. Data were from different animals during aging and subjected to one-way ANOVA followed by LSD as a post-hoc
Fasting blood insulin levels significantly increased during aging (repeated measures, p<0.05; longitudinal setting) with the significant increase occurring between 9 and 12 months (paired sample t test, p<0.05, Figure 14). After 12 months, the fasting insulin levels tended to remain relatively high, although not significantly different from 12 months. Fasting glucose levels did not change during aging (Figure 14).

Figure 14. Four-hour fasting plasma glucose and insulin levels. Repeated measures revealed no significant difference in glucose, but significant change (p<0.05) in insulin levels during aging. Paired sample t test was used to compare insulin levels at two different ages. Different letters denote significant difference (p<0.05).

Identification of plasma proteins

Overall, 86 protein spots representing the majority of the plasma proteins observed in this gel system, were identified by MS, MS/MS and LC/MS (Figure 15). Detailed MS and MS/MS or LC-MS/MS scores are listed in Table 15 and Table 16; Mw and pI of
these spots were listed in Table 17 of appendix A). Among these identified proteins, 13 showed significant changes in plasma levels during aging (repeated measures, p<0.01).

Figure 15. 2D gel reference map of identified proteins. Proteins in the red boxes are identified and if more than one spot is indicated, isoforms are numbered 1, 2, etc. In
order to simplify the labeling of protein and protein isoforms, two duplicate gel images are presented as A and B. Abbreviations: Mw: molecular weight; pI: isoelectric point; apoA4: apolipoprotein A-4; a2m: α-2 macroglobin; apoE: apolipoprotein E; TTR: transthyretin; MBP-C: mannose binding protein-C; Ig kappa: immunoglobulin kappa chain; Prx-2: peroxiredoxin-2; RBP-4: retinol binding protein-4; SAA-1: serum amyloid A-1, Hbb: hemoglobin β-chain.

**Plasma proteins that increased during aging**

The levels of six plasma proteins were significantly increased during aging in the longitudinal study (Figure 16 and Figure 15). The increase of these proteins during aging was confirmed in the cross-sectional study (data not shown). These proteins include three isoforms of immunoglobulin kappa chain (Ig kappa), of which spots 1 and 2 corresponded to Ig kappa variable III region (Ig kappa V-III) and spot 3 Ig kappa constant region (Ig kappa C). In addition, isoform 3 of transthyretin (TTR) and isoforms 2 and 3 of haptoglobin (Hp) also increased. Notice that the three Ig kappa chain isoforms did not change from 2 to 8 months (young to adult), but started to increase at 12 or 16 months (middle, old age). On the other hand, TTR isoform 3 and Hp isoforms 2 and 3 were increased as early as 4 or 8 months.
Figure 16. Plasma proteins that significantly increased in WT mice during aging (repeated measures, p<0.01). Protein intensities are log-transformed and presented in arbitrary unit (y axis). Protein isoform # corresponds to Figure 15.
Figure 17 shows the 3-D view of intensity of Ig kappa (spot 1) during aging. Notice that the spot was barely detectable from 2-8 months, but became apparent at 16 and 19 months.

Ig kappa V-III region (spot 1)

*Figure 17. 3-D view of the intensity of Ig kappa (spot 1) in WT mice at different ages. The spot intensity was converted into this topographic view of a peak using PDQuest. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.*
Similarly, Ig kappa spots 2 and 3 became detectable only after 12 months (*Figure 18* and *Figure 19*).

*Figure 18*. 3-D view of intensity of Ig kappa (spot 2) of WT mice at different ages. At the lower right corner is the original gel image of the spot, which is circled and indicated by an arrow.
Figure 19. 3-D view of intensity of Ig kappa (spot 3) of WT mice at different ages. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.

TTR isoform 3 was detectable as early as 2 months of age with a very low intensity. It continued to increase to 19 months when the spot became visually obvious (Figure 20).
Figure 20. 3-D view of intensity of TTR isoform 3 of WT mice at different ages. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.

Hp isoforms 2 and 3 were non-detectable at 2 and 4 months, increased during aging and had a relatively high intensity at 16 and 19 months (Figure 21).
Figure 21. 3-D view of intensity of Hp isoforms 2 and 3 of WT mice at different ages. At the upper right corner is the original gel image of the two spots, which are circled and indicated by arrows.

Plasma proteins that decreased during aging

Seven proteins were significantly decreased during aging in the longitudinal study (p<0.01, Figure 22). The decrease of these proteins was confirmed in cross-sectional study (data not shown). These proteins include peroxiredoxin-2 (Prx-2), five isoforms of albumin (isoforms 6-9 and 18), and serum amyloid protein-A1 (SAA-1). Notice that all these proteins had little change from 2 to 8 months (young to adult), but started to decline rapidly from 12 months (middle age) to 16 and 19 months of age.
**Figure 22.** Plasma proteins that significantly decreased in WT mice during aging (repeated measures, p<0.01). Protein intensities are log-transformed and presented in arbitrary unit (y axis). Protein isoform # corresponds to **Figure 15**.

Prx-2 started at relatively high levels but decreased after 8 months (**Figure 23**).

**Figure 23.** 3-D view of intensity of Prx-2 in WT mice at different ages. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.

Albumin (isoforms 6-9) all decreased from 8 months onward (**Figure 24**).
Figure 24. 3-D view of intensity of albumin isoforms 6-9 in WT mice at different ages. At the lower right corner is the original gel image of the four spots, which are circled and indicated by arrows.
Albumin isoform 18 decreased after 12 months (Figure 25).

*Figure 25. 3-D view of intensity of albumin isoform 18 of WT mice at different ages. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.*
SAA-1 decreased slightly but in a statistically significant manner (p<0.01) as mice aged. A topographic view of the spot intensity during aging is shown in Figure 26.

Figure 26. 3-D view of intensity of SAA-1 in WT mice at different ages. At the upper right corner is the original gel image of the spot, which is circled and indicated by an arrow.
Discussion

As mice aged, lean mass increased from young to middle age and did not change much from middle to old age; whereas fat mass increased at middle age, but started decreasing after 19 months (old age). As a result, body weights increased during aging with a slight weight loss after 19 months of age (Figure 13). A subset of these data (from 6 to 12 months) are in agreement with previous results in which mice were followed up to one year of age (Palmer et al., 2009). In humans, fat gain is associated with aging, but wasting at old age is also common. Unlike mice, wasting in humans happens with muscle as well fat loss (Fanciulli et al., 2009). This is likely due to species difference, but it is also possible that lean mass loss may be observed if mice are followed to ages older than 24 months. One note is that the sum of lean, fat and fluid mass is always smaller than body weight measured by a scale (comparing Figure 13 A and B). The reason for this discrepancy is that body composition was measured by an NMR analyzer which utilizes the nuclear magnetic resonance of hydrogen, resulting in certain tissues not being measured (i.g., bone mineral).

Another characteristic of human aging is reduced insulin sensitivity, which was found in mice as well. Mice in this study had normal fasting glucose levels throughout life, but increased insulin levels at middle and old age (Figure 14), suggesting decreased insulin sensitivity at these later ages. Similar trend of insulin levels going up during aging in WT mice was also reported previously (Coschigano et al., 2003). The onset of increased insulin coincided with fat mass gain, suggesting a possible role of fat in insulin resistance that is commonly known (Guy-Grand & Bour, 1972; P. L. Huang, 2009;
Mikhail, 2009; Muhlhauser & Smith, 2009). However, insulin did not decrease at old age when fat loss occurs, suggesting other mechanisms are responsible for insulin-resistance at old ages.

In order to uncover other biomarkers of aging, we used 2-DE as a proteomic approach to resolve mouse plasma proteins in the natural course of mouse aging. One problem in isolating plasma proteins is that a few species of abundant proteins occupy most of the protein mass (Figure 4 in chapter I) (N. Leigh Anderson & Anderson, 2002). Albumin, for example, comprises of 60% of all serum protein mass (Rothemund et al., 2003). The high-abundance proteins result in high background smearing on 2D gels (Thadikkaran et al., 2005). Relatively cheap, reproducible commercial kits are available for removal of human albumin from serum or plasma; however, no such kits are available for the treatment of mouse blood. To circumvent this problem, we used high concentration of polyacrylamide (15%) to resolve proteins with Mw below 45 kDa (Figure 9B), which is smaller than albumin (~70 kDa).

We have quantified and identified 86 spots in mouse plasma during normal aging. As can be seen from the gel profiles, these proteins represent the majority (in mass abundance) of all the proteins resolved (Figure 15). The protein distribution pattern on the 2D gel is comparable to a previous study that reported 57 identified mouse plasma proteins (Duan et al., 2004). We have discovered 13 spots corresponding to 6 distinct gene products that showed significant changes (repeated measures, p<0.01) as mice aged. These are described below.
Mouse TTR is in theory 13.6 kDa (without the signal peptide) with a pI of 5.77. Nine isoforms of TTR have been identified (Figure 15). The observed Mw of TTR identified on 2D gel is ~14 kDa for isoforms 4-9, ~21 kDa for isoform 3, and ~40kDa for isoforms 1 and 2 (Figure 15 and Table 17 in Appendix A). TTR is known to exist in human plasma in different forms due to PTMs such as phosphorylation, cysteinylation, dihydroxylation and glutathionylation (Hutchinson et al., 1996; Terazaki et al., 1998; Zheng Wang et al., 2004). In SWISS-2DPAGE database, human TTR is resolved as multiple spots on a 2D gel, including ~14 kDa and ~38 kDa forms, with the larger form thought to be multimers of TTR (database, 2009). Similar TTR isoforms have also been identified in mouse serum by (Duan et al., 2004). Also Okada et al from our group have reported similar forms of TTR (manuscript submitted).

We have found that total TTR levels (sum intensity of all identified TTR isoforms; this is not necessarily the total TTR in plasma as there may be TTR spots not identified on the gels. However, this is a measurement of ‘total’ TTR in this study) fluctuated but increased significantly (p<0.01) as mice reached old age (Figure 27). In particular, isoform 3 increased throughout aging (Figure 20).
Figure 27. Total protein levels of TTR and RBP-4 in WT mice during aging. ‘Total protein’ is defined as the sum of all isoforms. Total TTR and RBP-4 changed in a similar pattern during aging.

The majority of TTR in plasma is secreted from the liver and forms a homotetramer that binds and transports thyroxine (T4) and retinol binding protein-4 (RBP-4). TTR binding to T4 is thought to increase T4’s half life, facilitating its controlled release and preventing the toxic effects of high-concentration free thyroid hormone (Schussler, 2000). TTR also binds to RBP-4 to increase its half life by reducing kidney filtration. There are four RBPs with a primary role of binding and transporting retinol in both humans and mice. RBPs 1-3 are present intracellular whereas RBP-4 is found in the blood.

Interestingly, total RBP-4 levels at different ages were in parallel with those of total TTR (Figure 27), both fluctuating with an increasing trend toward old ages. Since TTR binds to RBP-4 and prevents its kidney filtration, this suggests that the sum of all isoforms of TTR and RBP-4 reflects to a large extend the total protein levels in plasma. RBP-4 is associated with obesity and insulin resistance (Haider et al., 2007; Kowalska et
Plasma TTR and RBP-4 both increase in type II diabetic subjects (Raila et al., 2007). In this regard, increased total TTR may reflect increased insulin resistance as mice aged. Interestingly, in both male and female humans, TTR levels also increase as a function of age (Figure 28C,D) (Robert et al., 1999a; Robert et al., 1999b). This trend of TTR increase resembles that of mouse aging in Figure 27.

Figure 28. Albumin and TTR levels during human aging. The closed circles (●) represent the observed median level. The lines represent the predicted median values. A and C, males; B and D, females. Adapted from (Robert et al., 1999b).

A condition called senile cardiac TTR amyloidosis has been found to be the primary cause of death in supercentenarians, people over 110 years old (Leslie, 2008). These
supercentenarians are usually free of cancer, cardiovascular diseases and neurodegenerative diseases. However, ‘clogging’ of blood vessels at autopsy has been found to be comprised of TTR amyloid in supercentenarians. Also, TTR can form plaques that deposit systemically (all over the body) (Ando, 2006). Finally, certain TTR mutants or variants have been found to be more susceptible to amyloid formation (Ando, 2006).

As mentioned earlier, TTR is modified by different PTMs. One specific PTM, a thiol conjugation, is found to be dependent on the age of a person and its increase is indicative of symptomatic amyloid disease (Suhr et al., 1999). Further, certain mutant TTR is more likely to be modified by thiol conjugation than the wild type (Suhr et al., 1999). Thus, PTMs of TTR may be related to amyloid formation and/or toxicity. Therefore, it will be interesting to identify the PTM responsible for isoform 3.

**Hp**

Hp is composed of α and β subunits that are cleaved from a prepropeptide and connected by disulfide bonds. Hp is ~31 kDa (without an 18-amino acid signal peptide). However, in 2-DE, the two subunits are disassociated by reduction and alkylation. The theoretical Mw of the α-subunit is ~9.5 kDa and the β-subunit is ~27 kDa. The spots observed in Figure 15 matches the prediction of the α-subunit of Hp, not the β, by MS/MS as well as LC-MS/MS (see Table 15 and Table 16 in Appendix A for details). It is known that β-subunit is glycosylated, but no report has confirmed glycosylation for the α-subunit. In the SWISS-2DPAGE database for human plasma, the Hp β-subunit resolves as multiple spots at ~45-48 kDa, which is beyond the limits of detection of our gel.
system. In a previously resolved mouse serum 2D system, Hp α-subunit is also reported at similar Mw (15 kDa) and pI (5+), whereas Hp β-subunit resolved at 42 kDa with pI more acidic than 5 (Duan et al., 2004). Hp β-subunit has not been identified from our gels perhaps because of this. However, since Hp is synthesized as one peptide which is then cleaved into two chains, presumably the level of α-subunit is representative of the full-length protein. Among the four Hp isoforms identified in our gels, isoforms 2 and 3 increased during aging.

Hp is mainly secreted by liver, but also by adipose and lung tissues. In case of hemolysis in blood vessels (as opposed to normal degradation of red blood cells in the spleen), Hp binds to hemoglobin to protect against heme-induced oxidative stress. Hp is also an acute phase protein (proteins that are induced in the liver by inflammation) highly induced in the liver by inflammation and injury (Dobryszycka, 1997). In elderly people, a higher concentration of Hp is correlated with infection or inflammation (Katz et al., 1990). It is found that in healthy aging population, serum concentration of Hp is negatively correlated with cognitive performance (Teunissen et al., 2003). In one proteomic study engaging 2-DE, Hp is found preferably in old aorta tissues but rarely in young, potentially indicating senescent vessels (Song et al., 1985).

In diabetes, acute phase proteins, including Hp, (Fleck, 1989) increase in the serum (McMillan, 1989). Hp has been known to be a major susceptibility gene in diabetic vascular complications in humans (Asleh & Levy, 2005). Different alleles in humans are thought to have different antioxidant efficiencies, resulting in different susceptibilities to diabetic nephropathy, retinopathy, and cardiovascular disease (Bessa et al., 2007;
Nakhoul *et al.*, 2007). As increased inflammation appears to be a common theme for both diabetes and aging (Dowling & Simmons, 2009), it is possible that Hp plays a role in both processes. Thus, increased Hp isoforms during normal aging observed in this study could indicate a state of increased inflammation at older ages. Hp was not detected by 2-DE at 2 months, consistent with a previous study which does not detect Hp in normal male (6 weeks old) mouse plasma until inflammation has been induced by burn injury or infection (Duan *et al.*, 2004).

*Ig kappa*

Ig kappa chain is an immunoglobulin light chain. Three spots have been identified as Ig kappa. Two of them were matched to Ig kappa variable region III (spots 1 and 2) and one to Ig kappa constant region (spot 3) (see *Figure 15B*). Spots 1 and 2 have sequences matched to multiple Ig kappa V-III, which is a variable region including multiple variants, for example, Ig kappa V-III region PC 7043 and region MOPC 63. The theoretical Mw of these Ig kappa V-III regions is about 12 kDa. Ig kappa C is 11.8 kDa with pI 5.23 in theory. All three spots are about 30 kDa on the gel, perhaps due to multimerization of Ig kappa chains. All three increased during aging, it possibly indicates that mice have undergone increased immune insults as they aged. Unfortunately, the nature of the Ig kappa chain is not known, so it is difficult to say if this is merely a reflection of increased concentration of immunoglobulins in old animals or if these are Igs targeting a specific antigen, or if the process reflects an infection or autoimmune disease.
Prx-2

Prx-2 has a theoretical Mw of 22 kDa and pI of 5.2. On the gel, it appears to be at the location of Mw 25 kDa and pI 6.1. The known PTMs of this protein include acetylation and phosphorylation (Swiss-Prot). We have found that Prx-2 decreased in normal aging.

Prx-2 is an anti-oxidant enzyme involved in reducing peroxide levels in cells. It is present in cytoplasm rather than being secreted. However, it is present in plasma at a relatively large concentration and has also been identified by 2-DE in mouse serum samples with similar Mw and pI (Guipaud et al., 2007); the same study shows that serum Prx-2 levels decreases after mice have been exposed to skin irradiation. Thus Prx-2 seems to be reduced by irradiation stress.

In C. elegans, Prx protects against oxidative stress and heat stress, as well as promotes longevity as evidenced by shortened lifespan of the loss-of-function mutant (Oľhová et al., 2008). A proteomic study using 2-DE identified Prx-2 as being induced in primary astrocyte cultures from young rats (1 month) but not adult or aged rats (9 and 24 months) after exposure to X-ray irradiation (Miura et al., 2007). Prx-2 inhibits mouse embryonic fibroblast senescence by inhibiting Ras-ERK-NFκB pathway (Han et al., 2006). Together, the evidence suggests that Prx-2 protects against oxidative stress and inhibits aging. Interestingly, its concentration decreased as mice aged in the current study, perhaps indicating reduced capacity to battle reactive oxygen species (ROS) at older ages.
**Albumin**

Full-length albumin has an Mw of ~70 kDa. As mentioned earlier, the high-concentration SDS-PAGE used in our 2-DE resolved proteins smaller than 45 kDa; therefore, full-length albumin would not be detected on these 2D gels. In fact, the albumin spots identified were all fragments. We have identified a total of 23 albumin fragments (*Figure 15B*). Isoforms 6-9 were ~24 kDa and matched to an N terminus fragment of albumin by MS/MS; isoform 18 was ~12 kDa and also matched to an N terminal fragment of albumin by MS/MS (Table 15 in Appendix A). In addition, other spots of different sizes were also identified as albumin, including isoforms 1-4 (32 kDa), isoform 5 (28 kDa), isoform 10 (24 kDa), isoforms 11-17 (16 kDa), isoforms 19 and 20 (10 kDa) and isoforms 21-23 (8 kDa). Of these, isoforms 5, 14-17 and 20 were C-terminal fragments; isoforms 1-4, 6-10, 11-12, 18, 19 and 21-23 were N-terminal fragments (Table 15). Each fragment is represented in albumin in *Figure 29*. 
Figure 29. Schematic representation of albumin fragments. Mouse full length albumin amino acid sequence is displayed. As shown, each color-coded box represents matched sequence by MS/MS of a particular albumin fragment. Numbers in the boxes represent the isoform number of albumin consistent with Figure 15.

Small Mw albumin fragments have also been found in mouse serum by 2-DE (Duan et al., 2004) and in human serum as reported in the SWISS-2DPAGE database (database, 2009). In NCBInr protein sequences database (NCBInr), there are several entries reporting mouse serum albumin fragments, including a 20.4 kDa N-terminal fragment (the Mw is calculated from primary sequence, ACCESSION: CAC81903, GI:26986064)
and a 7.8 kDa fragment from the internal sequence (CAA31458.1 GI:899334). In addition, there are numerous entries in NCBInr of albumin fragments in a wide range of species (NCBInr). Thus, it appears that it is quite common for animals to have plasma albumin existing as multiple forms with different sizes.

We have found that five albumin fragments decrease as mice aged. It is not clear how fragmentation of albumin in plasma is regulated. One study found a proteolytic activity degrading albumin in urine and identified α−1 antitrypsin as an anti-proteolytic protein to inhibit albumin fragmentation (Magistroni et al., 2009).

In humans, a lower serum albumin concentration is found in the elderly (Figure 28A,B) (Gom et al., 2007; Nakamura & Miyao, 2007; Robert et al., 1999a; Robert et al., 1999b). In rats, serum albumin concentration increases from 3 months to 7 months of age, then decreases in 12 and 20 months (Barber et al., 1995). Interestingly, the five mouse albumin fragments observed in this study showed little change from 2 to 8 months, but decreased from 8 months to 19 months (Figure 22, Figure 24 and Figure 25). However, the reduced levels of these albumin fragments were not necessarily due to the reduction of total albumin concentration as a function of mouse age because some of the other albumin fragments actually increased during aging (not reported because p values were not below cut-off value of 0.01). In addition, only albumin fragments smaller than 45 kDa were resolved on the gels. It is very possible that while total albumin in blood decreased at old age, certain fragments increased whereas others decreased or remained the same. Regardless, the five albumin fragments identified here
were consistent in both longitudinal and cross-sectional samples and significantly (p<0.01) decreased with age.

The function of albumin mainly involves transportation of small molecules such as water, Ca^{2+}, Na^+, K^+, fatty acids, hormones, bilirubin and drugs and regulation of colloidal osmotic pressure of blood. In terms of age-related diseases, higher serum albumin levels are found to be associated with reduced cardiovascular mortality and coronary heart disease and stroke incidence (Gillum, 2000). Albumin is also considered an antioxidant protein that scavenges ROS (Halliwell & Gutteridge, 1990), a negative acute phase protein that is decreased by inflammation (Fleck, 1989), and a common nutritional marker with decreased levels indicating malnutrition.

**SAA-1**

SAA-1 is a 12 kDa protein secreted by liver. The observed Mw of this protein on the gel is 11.5 kDa. It showed a significant decrease as mice aged. SAA-1 is found to be associated with high-density lipoprotein (HDL). It is known that lipid profiles change during aging (Bittner, 2003; Karlamangla et al., 2004; Klag et al., 1993; Kronmal et al., 1993). For example, non HDL cholesterol increases in the elderly (Bittner, 2003) and HDL decreases during aging (Wilson et al., 1994). Total cholesterol is positively correlated with increased mortality at age 40, but this correlation becomes increasingly less obvious at ages 50, 60 and 70, and at age 80, total cholesterol is negatively correlated with mortality (Kronmal et al., 1993). HDL is considered the ‘good’ cholesterol that is associated with decreased cardiovascular disease risks and better cognitive function (Atzmon et al., 2002), and shows increased levels in centenarians (Nir et al., 2001). Low-
density lipoprotein (LDL), on the other hand, is considered ‘bad’ cholesterol and is associated with increased risks for cardiovascular disease (Bittner, 2003). Since SAA-1 is an apolipoprotein for HDL, the decrease of SAA-1 may therefore reflect or contribute to aging by altering the lipid profile.

Other than lipid transportation, SAA-1 is also an acute phase protein induced by cytokines in response to infection or inflammation (Fleck, 1989). Interestingly, in humans, SAA-1 is also the precursor of reactive amyloid fibrils in type AA amyloidosis, a condition caused by deposition of insoluble fibrillar amyloid proteins (a degraded N-terminus fragment of SAA) in the extracellular space in many organs and tissues (Merlini & Bellotti, 2003). The chronic high expression of SAA in prolonged inflammation is considered to be essential for amyloidogenesis (Yamada, 2006). Several alleles were discovered that had different susceptibilities to amyloid formation, e.g., SAA1.1 was more susceptible than SAA1.5 (van der Hilst et al., 2008). To our knowledge, a role of SAA-1 in aging has not been reported.
Conclusion

Longitudinal analysis of mouse plasma proteome by 2-DE revealed two important results. 1) Many proteins exist as multiple isoforms. 2) In normal male mice, certain plasma proteins undergo significant changes during aging. These proteins and/or their isoforms, therefore, are biomarkers of mouse aging (Table 6).

Table 6. Biomarkers of aging in WT mice

<table>
<thead>
<tr>
<th>Increased during aging ↑</th>
<th>Decreased during aging ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp isoforms 2 and 3</td>
<td>Prx-2</td>
</tr>
<tr>
<td>Two Ig kappa V-III isoforms (spots 1 and 2); one Ig kappa C isoform (spot 3)</td>
<td>Albumin isoforms 6-9 and 18</td>
</tr>
<tr>
<td>TTR isoform 3</td>
<td>SAA-1</td>
</tr>
</tbody>
</table>

Spot # corresponds to those labeled in Figure 15.

The plasma proteins described in this report are involved in many aspects of aging or age-related diseases. Prx-2, Hp and albumin are antioxidant proteins that protect against oxidative stress, which is an important aspect of aging. SAA-1 and TTR have primary roles in transportation/metabolism of lipids or RBP-4 and T4, but are also involved in amyloid formation in blood vessels or tissues, a phenomenon associated with old age. In addition, albumin is involved in cardiovascular health and TTR in insulin resistance, both contributing heavily to morbidity and mortality at old age. Hp, SAA-1, albumin and TTR are acute phase proteins that indicate inflammation, which is likely associated with aging.
Of particular interest is that the different isoforms of the same protein did not always show the same profile relative to each other or with the total protein levels during aging (i.e., albumin and TTR). That is, some isoforms may increase, while others decrease. It is possible that different isoforms actually have different functions, contributing differentially to aging. Alternatively, isoform composition can affect the function of a given protein, and changes of the composition may be physiologically important and therefore reflective of aging. It is intriguing to study the PTMs responsible for the apparent isoforms. These plasma proteins can be modified in the secretory pathway inside the cell (e.g. hepatocyte) or after they are released into circulation. The regulation of generation of these PTMs is the underlying phenomena that future studies will address. Unraveling the PTMs and their regulation by aging may shed light on some of the molecular mechanisms of aging. Extension of these results to humans will be of obvious importance.

Are these proteins merely the markers of the aging process or do they actually play a causative role in aging? The functions of many of these proteins are not completely known; even less or nothing is known about the specific isoforms detected on the 2D gels. Future studies are needed to investigate the roles of these proteins/isoforms, the nature of their PTMs, and their functions during aging. With the elucidation of the mechanisms responsible for the phenotypic changes, we may be able to use these biomarkers as potential therapeutic targets that may ultimately combat aging, thereby increasing the lifespan and perhaps more importantly healthspan of an individual.
CHAPTER IV. BIOMARKERS OF CHRONIC GH ACTION: PROTEOMIC ANALYSIS OF PLASMA FROM MALE BGH TRANSGENIC AND CONTROL MICE

Abstract

Bovine growth hormone (bGH) transgenic mice are giant, lean, insulin-resistant and possess life spans shorter than wild type (WT) mice. To discover biomarkers indicative of GH action and perhaps indicators of an accelerated aging phenotype, we analyzed mouse plasma proteins of different ages by two-dimensional electrophoresis (2-DE) and identified proteins of interest by mass spectrometry (MS) and MS/MS. We found many proteins whose plasma levels changed between bGH and control mice. Several of these proteins existed as multiple isoforms presumably due to post-translational modifications (PTMs). Specifically, apolipoprotein (apo) E, haptoglobin (Hp) and mannose-binding protein-C increased significantly (p<0.01) in bGH mice versus controls; whereas transthyretin, apoA1 and retinol-binding protein-4 (RBP-4) significantly decreased. In addition, RBP-4 and alpha-2 macroglobulin exhibited isoform-specific changes in bGH mice, suggesting GH regulations of these proteins at PTM level. Importantly, two isoforms of Hp and clusterin exhibited a significant interaction between genotype and aging, increasing markedly during bGH aging with no change or less dramatic changes during WT aging. Thus, these two proteins are biomarkers reflecting an age-dependent difference in bGH mice.
Introduction

Growth hormone (GH) has been shown to be involved in the regulation of longitudinal growth, lactation and metabolism (Kopchick & Andry, 2000). GH acts on various tissues including bone, muscle, liver and fat. GH induces the expression of a potent growth factor called insulin-like growth factor-1 (IGF-1), which acts on target tissues through the IGF-1 receptor (R) via endocrine/paracrine or autocrine actions. The GH/IGF-1 axis has been implicated in aging, as an attenuated GH/IGF-1 axis has been found to be beneficial for prolonged lifespan in a wide range of organisms (Andrzej Bartke, 2005; Bonafè & Olivieri, 2009; Brown-Borg, 2009; Holzenberger et al., 2003; Tatar et al., 2003).

The physiological effect of GH can be seen in transgenic mouse models that over-express GH. Bovine (b) GH transgenic mice have markedly elevated serum levels of IGF-1, are gigantic, lean, insulin-resistant (Fernando P. Dominici et al., 1999a; Valera et al., 1993) and die prematurely due to liver, kidney and heart problems (A. Bartke, 2003; Berryman et al., 2004; Knapp et al., 1994; Quaife et al., 1989). In addition, GH transgenic mice have decreased reproductive life span, increased astrogliosis (abnormal increase of astrocyte number due to death of nearby neurons), and premature changes in cognitive function, hypothalamic neurotransmitter turnover, and plasma corticosterone levels (A. Bartke, 2003).

Serum/plasma samples are routinely used for disease diagnosis. There are an estimated 10,000 proteins in human plasma and 1175 distinct proteins/peptides have been identified (N. Leigh Anderson et al., 2004). In mice, 12,300 unique peptides that
originate from 4567 unique proteins have been identified through a multidimensional fractionation and tandem mass spectrometry (MS) approach (Hood et al., 2005). Plasma, therefore, has a huge repertoire of proteins, some of which may be potential markers of GH action.

In this study, plasma proteins of bGH mice were compared to wild type (WT) littermates during aging using a two-dimensional electrophoresis (2-DE) technique coupled with MS and MS/MS for protein identification. In this study, 29 novel plasma proteins/isoforms involved in the action of GH have been identified. These results may be beneficial for better understanding the pathphysiology of acromegaly, as well as in providing a background for the discovery of biomarkers of GH action in humans especially when recombinant human (rh) GH is misused or abused. RhGH doping is currently an important issue on the agenda of the World Anti-Doping Agency (WADA) (WADA, 2009).
Materials and Methods

Experimental animals

The bGH transgenic mice were generated by injecting a linearized plasmid containing a metallothionein transcriptional regulatory region (promoter) driven bGH cDNA into the pronucleus of C57BL/6J embryos as described previously (Berryman et al., 2004; McGrane et al., 1988; Wagner et al., 1981; Yang et al., 1993). WT littermates were used as control animals. Male bGH mice (n=9) and WT (n=8) in the background of C57 BL/6J were followed during aging at 2, 4, 8, 12, 16 and 19 months of age. These ages represent post-puberty (2 months), young adult (4 months), middle age (8 and 12 months) and ‘old age’ (16 and 19 months) in mice (Richard A. Miller & Nadon, 2000).

Mice were housed 2-3 per cage at room temperature (22 °C) in a 12-hour light, 12-hour dark cycle, fed ad lib with a standard chow diet. Animal protocols were approved by Ohio University’s Institutional Animal Care and Use Committee.

Body composition measurement

Body composition was measured at 9, 13 and 19 months using mq 7.5 NMR analyzer, the Bruker Minispec (The Woodlands, TX) as described previously (List et al., 2009; Palmer et al., 2009). The data are presented as absolute mass (gram, or g) as well as percentage of body weight (%) of lean mass, fat mass and fluid mass. Each animal at each age was measured twice and the mean value used.

Plasma IGF-1 measurement

Blood was collected with heparinized capillary tubes by tail tip clipping following heat lamp exposure. Whole blood was centrifuged at 7000 x g for 10 minutes at 4 °C and
the resulting plasma was stored at -80 °C. Plasma IGF-1 levels were determined using the DSL-10-29200 mouse/rat IGF-1 ELISA kit purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX).

**Fasting glucose and insulin measurements**

Mice were fasted for 4 hours and bled at 3 PM. Blood glucose was measured using a ONE TOUCH glucometer from Lifescan (Milpitas, CA). Plasma was collected as described above and insulin levels measured using an ultrasensitive rat/mouse insulin ELISA kit (ALPCO, Windham, NH).

**2-DE and quantification of proteins**

See chapter III.

**Statistical analysis**

All statistical analyses were performed using SPSS 14.0 software (Chicago, IL). Data for IGF-1 and body composition were subjected to t test with significance value p<0.05. Data for body weight, fasting glucose and insulin were subjected to repeated measures with significance value p<0.05. For protein intensity, the log-transformed data were subjected to repeated measures for five different age points (2, 4, 8, 12 and 16 months), with genotype as a fixed factor (p<0.01). The data at 19 months was excluded from repeated measures because 5 bGH mice died at 19 months. For genotypic difference at each age, an independent t test was used (including at 19 months). Data of bGH mice (n=4) at 19 months was also graphed to show the trend of any change. All data were presented as mean ± SEM.
Protein identification by mass spectrometry (MS), MS/MS, and liquid chromatography (LC)-MS/MS

See chapter III.
Results

Body weight and body composition

Whereas WT mice showed steady body weight gain from weaning (3/4 months) to 19 months, bGH mice reached a weight plateau at 9 months and started losing weight thereafter (Figure 30A). However, throughout their lifespans, with the exception of ¾ months and 19 months, bGH mice had significantly greater weights than WT at each age point (p<0.00001).

Body composition was examined at 9, 13 and 19 months. WT and bGH mice showed marked differences in lean, fat and fluid mass at each age (Figure 30B, C, D; Figure 31A). At each age, bGH mice had significantly higher lean mass than WT (p<0.0001) and significantly lower fat mass than WT animals (p<0.01). At 9 and 13 months, bGH mice had significantly increased fluid mass than WT (p<0.001 and p<0.01, respectively), but no significant difference at 19 months. Noteably, whereas WT mice had significant age-related fat mass gain (paired sample t test, p<0.05), bGH mice showed significant fat mass loss during aging (paired sample t test, p<0.01). Also, WT mice showed increased fluid mass during aging (paired sample t test, p<0.05) whereas bGH mice showed no significant change.

As shown above, in terms of absolute mass, bGH mice had an increase in lean and fluid mass (except at 19 months) and reduced fat mass compared to WT. When data in grams were converted to percentage of body weight, bGH mice still had increased lean% and decreased fat % compared to WT mice but fluid % became not significantly different between genotypes (Figure 31B).
Figure 30. Body weight and composition of bGH and WT mice during aging. A, body weight change during aging. One-way, repeated measures revealed a significant change of body weight during aging \((p<10^{-38})\), a significant genotype difference \((p<10^{-10})\) and a significant interaction between aging and genotype \((p<10^{-9})\). B, lean mass. C, fat mass. Paired sample t test revealed that WT mice had fat mass gain from 9 to 13 months \((p<0.05)\) whereas bGH had decreased fat mass \((p<0.01)\). D, fluid mass. Paired sample t test revealed that WT mice had fluid mass gain from 9 to 13 months \((p<0.05)\) whereas bGH had no change. * \(p<0.01\), ** \(p<0.001\), *** \(p<0.0001\) comparing of bGH to WT of the same age by t test.
Figure 31. Body composition of bGH and WT mice during aging. A, stacking bar graph of body composition in bGH and WT mice. B, normalized body composition as percentage of body weight. * p<0.01, ** p<0.001, comparing of bGH to WT of the same age.

**IGF-1, fasting plasma insulin and glucose levels**

At 6 months, bGH had significantly higher IGF-1 levels than WT mice (p<0.0001, Figure 32A). According to one-way repeated measures, fasting insulin levels were changed by aging significantly (p<0.01), but not by genotype. There was a significant interaction between aging and genotype (p<0.01, Figure 32B). bGH mice had an initial increased levels of insulin (from 3 to 5 months) and then they decreased at older ages. At 3 months, bGH mice had higher insulin than WT (p<0.05), however, from 12 months onward, bGH mice had significantly lower insulin levels than WT (p<0.05 for 12 months, p<0.01 for 16 months, and p<0.05 for 19 months). For fasting glucose levels, a
significant aging effect (p<0.01) and a genotype effect (p<0.001) as well as an interaction between aging and genotype (p<0.001) were observed (Figure 32C). The bGH mice had comparable levels of glucose with WT mice at young ages; however, from 12 to 19 months, bGH mice had significantly lower glucose levels than WT mice (p<0.01, Figure 32C).

Figure 32. IGF-1, insulin and glucose levels. A, IGF-1 levels at 6 months of age. B, 4-hour fasting insulin levels during aging. One-way repeated measures showed a significant aging effect (p<0.01) and a significant interaction between aging and genotype (p<0.01), with no genotype difference. C. 4-hour fasting glucose levels during aging. One-way repeated measures showed a significant aging effect (p<0.01) and a genotype effect (p<0.001) as well as an interaction between aging and genotype (p<0.001). #p<0.05, *p<0.01, **p<0.001, ***p<0.0001 comparing of bGH to WT of the same age by t test.
Detection of plasma proteins in bGH mice versus WT controls by 2-DE

Plasma proteins of WT and bGH mice at six different ages were analyzed (see Figure 85, Figure 86 and Figure 87 in Appendix B for original gel images). A number of proteins changed significantly in bGH mice using one-way repeated measures. Thirteen proteins increased while fourteen decreased significantly in bGH mice compared to WT controls, and 2 proteins (haptoglobin (Hp) and clusterin) showed a significant interaction between genotype and aging (p<0.01, Figure 33).

Proteins upregulated in bGH versus WT are numbered in red (# 1-13) in Figure 33, including all five isoforms of apolipoprotein E (apoE, # 2, 4-7), two isoforms of α-2 macroglobulin (a2m) (# 1 and 3), mannose binding protein-C (MBP-C, # 8), one isoform of retinol-binding protein-4 (RBP-4, # 9), and all four isoforms of Hp (# 10-13). Proteins downregulated in bGH mice are numbered in purple (# 14-28) in Figure 33, including eight isoforms of transthyretin (TTR, # 14, 20, 22-27), two isoforms of a2m (# 15 and 16), three isoforms of apolipoprotein A1 (apoA1, # 17-19) and one isoform of RBP-4 (# 21). Two isoforms of clusterin (spots 28 and 29) and all four isoforms of Hp (spots 10-13) showed significantly different aging patterns between bGH and WT mice, which are labeled with circles in Figure 33. The quantification of fold change in these proteins is listed in Table 7.
Figure 33. Plasma proteins up or downregulated in bGH compared to WT mice (p<0.01). Proteins are marked by green crosses for their locations on the gel and numbered 1-13 in red (upregulated proteins), and 14-27 in purple (downregulated). Spots in circles (spots 10-13, 28 and 29) are proteins that had a significant interaction between genotype and aging (p<0.01).
Table 7. Quantification of proteins up or downregulated in bGH mice

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein ID</th>
<th>Isoform #</th>
<th>Ratio of bGH/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2m</td>
<td>1</td>
<td>↑3.8</td>
</tr>
<tr>
<td>2</td>
<td>apoE</td>
<td>1</td>
<td>↑3.0</td>
</tr>
<tr>
<td>3</td>
<td>A2m</td>
<td>2</td>
<td>↑1.9</td>
</tr>
<tr>
<td>4</td>
<td>apoE</td>
<td>2</td>
<td>↑6.2</td>
</tr>
<tr>
<td>5</td>
<td>apoE</td>
<td>3</td>
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<tr>
<td>6</td>
<td>apoE</td>
<td>4</td>
<td>↑6.7</td>
</tr>
<tr>
<td>7</td>
<td>apoE</td>
<td>5</td>
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</tr>
<tr>
<td>8</td>
<td>MBP-C</td>
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<td>↑8.2</td>
</tr>
<tr>
<td>9</td>
<td>RBP-4</td>
<td>4</td>
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<td>Hp</td>
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<td>11</td>
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<td>3</td>
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<td>Hp</td>
<td>Sum</td>
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<tr>
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<td>TTR</td>
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<tr>
<td>15</td>
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<tr>
<td>17</td>
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<tr>
<td>18</td>
<td>apoA1</td>
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</tr>
<tr>
<td>19</td>
<td>apoA1</td>
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</tr>
<tr>
<td>20</td>
<td>TTR</td>
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</tr>
<tr>
<td>21</td>
<td>RBP-4</td>
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<tr>
<td>22</td>
<td>TTR</td>
<td>4</td>
<td>↓0.43</td>
</tr>
<tr>
<td>23</td>
<td>TTR</td>
<td>5</td>
<td>↓0.38</td>
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<tr>
<td>24</td>
<td>TTR</td>
<td>6</td>
<td>↓0.45</td>
</tr>
<tr>
<td>25</td>
<td>TTR</td>
<td>7</td>
<td>↓0.36</td>
</tr>
<tr>
<td>26</td>
<td>TTR</td>
<td>8</td>
<td>↓0.29</td>
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<tr>
<td>27</td>
<td>TTR</td>
<td>9</td>
<td>↓0.19</td>
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<tr>
<td>Total</td>
<td>TTR</td>
<td>Sum</td>
<td>↓0.40</td>
</tr>
<tr>
<td>Total</td>
<td>RBP-4</td>
<td>Sum</td>
<td>↓0.75</td>
</tr>
</tbody>
</table>

Spot # corresponds to Figure 33; isoform # corresponds to Figure 15. The ratio bGH/WT is calculated as the mean value of bGH/mean value of WT (pooled values of all ages). ‘↑’ means up-regulation and ‘↓’ means down-regulation in bGH versus WT mice. Total protein is defined as the sum of all identified isoforms of a given protein.

All five isoforms of apoE increased dramatically in bGH mice at all ages compared to age-matched WT controls (Figure 34 and Figure 35). These isoforms did not show significant changes during aging at a significance level p<0.01. However, total apoE, the sum of all isoforms, decreased significantly during aging in bGH (p<0.001) and WT mice.
(p<0.05), with total apoE levels significantly higher (more than 6 fold) in bGH mice (p<0.001).

Figure 34. Comparison of apoE isoforms in WT and bGH mice of different ages. A, cropped image of one representative gel from WT and bGH at each age. The red arrows point to individual apoE isoforms of mice at 4 months. B, 3-D view of apoE isoforms in one WT and one bGH mouse at 8 months of age. Spot intensity was converted into topographic view of a peak using PDQuest. The spot # corresponds to Figure 33.
ApoE increased in bGH compared to WT mice at all ages. Individual isoforms as well as total apoE are quantified at different ages in bGH and WT mice. Spot # corresponds to Figure 33. Total apoE is calculated as the sum intensity of all apoE isoforms. The Y axis represents log-transformed intensity.

Spot 8, an MBP-C isoform, increased in bGH mice significantly (p<0.01, Figure 36). It also showed a significant change relative to age in both genotypes (p<0.01), increasing from young to middle age and decreasing in old mice.
Figure 36. One isoform of MBP-C increased in bGH compared to WT mice. The spot number corresponds to that in Figure 33. A, MBP-C levels (log-transformed intensity) during aging in bGH and WT mice. B, 3-D view of the intensity of MBP-C from one WT and one bGH mouse at 12 months. At the upper right corner is the original gel image of the spot, which is circled and is indicated by an arrow.

All four Hp isoforms increased in bGH mice (Figure 37, Figure 38 and Figure 39A). All isoforms showed a significant increase in bGH mice during aging (p<0.01), but only spots 11 and 12 increased as a function of age in WT mice (p<0.01). All Hp isoforms increased during aging to a greater extent in bGH mice, thus showing a significant interaction between genotype and aging (p<0.01). Hp was not detectable in WT or bGH mice at 2 and 4 months, but became detectable after 8 months, with increasing levels found during aging. This occurred more so in bGH than WT mice. Total Hp, the sum of all isoform intensities, increased during aging (p<0.01 for WT and p<0.001 for bGH), with similar levels at 2 and 4 months but higher levels in bGH mice at later ages (p<0.01 for interaction) and overall higher levels in bGH mice (p<0.01).
Rapid age-related increase of Hp in bGH compared to WT mice

Figure 37. Hp in bGH and WT mice. Individual isoform as well as total Hp are quantified at different ages in bGH and WT mice. Spot # corresponds to Figure 33. Total Hp is calculated as the sum intensity of all Hp isoforms. The Y axis represents log-transformed intensity.
Figure 38. 3-D view of four isoforms of Hp (spots 10-13) and one isoform of RBP-4 (spot 9). The spot # corresponds to Figure 33.
Eight TTR isoforms decreased in bGH mice at all ages compared to age-matched controls (Figure 39, Figure 40 and Figure 43B). Spots 22 and 25 (Figure 40) also showed a significant increase during aging in both genotypes (p<0.01). The other isoforms did not change significantly with age. Total TTR, i.e., the sum of all isoform intensities, increased with age in both WT and bGH mice (p<0.01 for WT and p<0.001 for bGH), with a greater increase of the levels in bGH mice, although they were always lower in bGH than WT mice (p<0.001).

**Figure 39.** Comparison of TTR and Hp isoforms in WT and bGH mice at different ages. A, cropped image of one representative gel from WT and bGH at each age. Arrows indicate individual isoforms of TTR and Hp as labeled. The spot # corresponds to Figure 33. For each gel image, spots to the left of the dashed vertical line are Hp and those to the right are TTR.
right are TTR. B, 3-D view of TTR isoforms (spots 22-26) in one WT and one bGH mouse at 8 months of age.

Figure 40. TTR decreased in bGH compared to WT mice at all ages. Eight individual isoform as well as total TTR are quantified at different ages in bGH and WT mice. Spot # corresponds to Figure 33. Total TTR is calculated as the sum intensity of all TTR isoforms. The Y axis represents log-transformed intensity.
Three isoforms of apoA1 decreased in bGH compared to WT mice; no significant changes were seen during aging (Figure 41). The other three isoforms of apoA1 (shown in Figure 15 in chapter III) did not show a difference between genotypes or during aging. Total apoA1, the sum of all isoform intensities, also decreased (p=0.011). Thus, apoA1 was not changed by age and showed only a genotype difference.

Figure 41. ApoA1 decreased in bGH compared to WT mice. A, three isoforms of apoA1 decreased significantly (p<0.01) and total apoA1 also decreased (p=0.011). Total apoA1 is calculated as the sum intensity of all identified apoA1 (see Figure 15 for all apoA1 isoforms). The Y axis represents log-transformed intensity. B, 3-D view of apoA1
isoforms in one WT and one bGH mouse at 8 months of age. The spot # corresponds to Figure 33.

One isoform of RBP-4 (spot 9) decreased, whereas another one (spot 21) increased in bGH compared to WT mice (Figure 42 and Figure 38). However, total RBP-4 decreased in bGH mice versus WT mice. Both isoforms also showed significant fluctuation (decreased at middle age but increased again at old age) during aging in both genotypes (p<0.01). Total RBP-4, sum of all RBP-4 isoforms, fluctuated similarly in both genotypes during aging (p<0.001 for both), with the levels in bGH always lower than WT (p<0.001).

Figure 42. RBP-4 showed isoform-specific change in bGH compared to WT mice. A, quantification of spot intensities. Total RBP-4 is calculated as the sum intensity of all identified RBP-4 (see Figure 15 for all RBP-4 isoforms). The Y axis represents log-
transformed intensity. B, 3-D view of intensity of RBP-4 from one WT and one bGH mouse at 12 months. Arrows indicate the 3-D peak as well as the original gel image corresponding to the spot. The spot # corresponds to Figure 33.

A2m was identified as seven isoforms with similar Mw but pIs ranging from 5.6 to 7 (Figure 15 in chapter III). Of these, spots 1 and 3 increased, whereas spots 15 and 16 decreased in bGH mice (Figure 43 and Figure 44). None of the isoforms showed significant changes during aging. Total a2m did not change during aging or between genotypes.

Figure 43. Comparison of a2m isoforms in WT and bGH mice at different ages. A, cropped image of one representative gel from WT and bGH at each age. Arrows indicate individual isoforms. B, 3-D view of intensity of a2m isoforms from one WT and one bGH mouse at 8 months. The spot # corresponds to Figure 33.
A2m showed isoform-specific changes in bGH mice

Figure 44. A2m showed isoform-specific changes in bGH compared to WT mice. Spot # corresponds to Figure 33. The Y axis represents log-transformed intensity.

Two isoforms of clusterin increased markedly during aging in bGH but not WT mice (Figure 45 and Figure 46). Similar trend was found in other four isoforms but did not reach significance level of 0.01, therefore not shown. Total clusterin, the sum of all isoform intensities, did not change in WT, but increased significantly in bGH mice (p<0.001), with lower levels than WT at young ages but higher levels than WT at later ages. Both spots 28 and 29 as well as total clusterin showed marked increase in bGH mice during aging, with no significant change during WT aging, resulting in lower levels of clusterin when young, but higher levels in older bGH versus WT mice.
Figure 45. Clusterin showed significant interaction between genotype and aging. A, cropped image of one representative gel from WT and bGH at each age. Of the six isoforms shown in circles, spots 28 and 29 showed significant interaction between genotype and aging (p<0.01). Arrows indicate these two spots in all gels. Other 4 isoforms showed similar changes without reaching a significant threshold. Spot # corresponds to Figure 33. B, quantification of clusterin in bGH and WT mice during aging. The Y axis represents log-transformed intensity.
Figure 46. 3-D view of clusterin isoforms.
Discussion

*Body composition, IGF-1 and insulin sensitivity*

BGH mice had increased IGF-1, body weight, lean and fluid mass, and reduced fat mass, all consistent with features of acromegaly in humans (Chanson & Salenave, 2008). These data suggest that bGH mice are a good model to study acromegaly.

BGH mice had similar body weight as WT at weaning (3/4 month), suggesting that the growth-promoting effect of GH is not manifested before 3 weeks of age. Close examination of the mice at different ages revealed that from 3/4 to 19 months, bGH mice reached a weight plateau at 8 months and started losing weight after 9 months, whereas WT mice did not show weight loss till 19 months. Weight loss, therefore, could be a rough estimation of getting ‘old’. By this notion, WT mice started getting ‘old’ at 19 months at which age they started losing weight (chapter III). The early-onset weight loss of bGH mice reflects their shortened lifespan. In fact, 5 out of 9 bGH mice died at shortly before the age of 18 months. This early death rate in GH transgenic mice has been reported previously (Berryman et al., 2004; McGrane et al., 1988; Steger et al., 1993).

As expected, bGH mice had significantly higher weight than WT mice of the same age throughout their lifespan, manifesting the potent effect of GH in growth promotion and anabolism. WT had steady levels of lean mass from 9 months (middle age) to 19 months (old), whereas bGH mice showed no change of weight and even a slight decrease at 19 months. At each age, bGH had significantly higher lean mass than WT, showing GH’s anabolic action on lean mass. WT mice also had increased fat mass during aging (up to 19 months); in contrast, bGH had decreased fat mass as a function of age. In
chapter III of this dissertation, WT mice started losing fat and weight at 19 months; it seemed that bGH mice started to decline from 9 months, perhaps reflecting the accelerated aging in this mouse model. The fluid mass increased in WT throughout 19 months, but did not change in bGH mice. However, at each age, bGH mice had significantly higher fluid mass, perhaps due to the fluid retention effect of GH (Carroll & Van den Berghe, 2001; Clemmons et al., 1987; O'Sullivan et al., 1994). GH and IGF-1 promote fluid retention by stimulating renin-angiotensin-aldosterone system, which is central to fluid homeostasis (J. Moller et al., 1999). One cautionary note is that when normalized to body weight, fluid% remained the same in bGH and WT mice. The larger error bar at 19 months was probably due to the reduced sample size (n=4). Curiously, there may be a problem with urination in the very old bGH mice, since at dissection a huge, full bladder was noted (unpublished observation). It is interesting that in WT mice, fluid mass continued to increase from 19 to 24 months (chapter III), but remained unchanged in bGH mice from 9 to 13 months, possibly suggesting that the normal age-related fluid retention in mice is affected by high levels of GH. Together, body composition data suggest that for WT mice, 19 months is an ‘indicator’ for old age; however this ‘indicator’ is much earlier in bGH mice where 9 months can be considered old. Indeed, the mean lifespan is 409 ± 45 days (about 14 months) for bGH mice versus 602 ± 34 days (about 21 months) for WT in this mouse line (mentioned in (Berryman et al., 2004) as unpublished observations, and similar in other hGH or bGH lines (McGrane et al., 1988; Steger et al., 1993). The body composition data before 13 months in this
study was consistent with a previous study, in which bGH and WT mice were followed to one year of age (Palmer et al., 2009).

As previously shown (chapter III), fasting insulin levels increased in WT mice during aging; however, insulin levels increased only from 3 to 5 months in bGH mice. Although this study only looked at body composition after 9 months, a previous study reported that from 1.5 to 5 months of age, bGH mice had increased fat mass as a function of age similar to WT mice and strikingly more absolute fat mass than age-matched WT animals (Palmer et al., 2009). Therefore, the increase in insulin with age may reflect fat increase in bGH mice during this time period. From 1.5 to 3 months of age, bGH mice also have a greater percentage of fat mass than WT, but the normalized fat mass become less than WT after 3 months (Palmer et al., 2009). This may be a part of the reason why bGH mice had higher insulin levels than WT at 3 months but was not higher at 5 and 9 months when they started showing less normalized fat than WT mice. This implies a role of fat in insulin levels as has been shown numerous times (Guy-Grand & Bour, 1972; P. L. Huang, 2009; Mikhail, 2009; Muhlhausler & Smith, 2009).

BGH mice are known to be hyperinsulinemic, however, most of the literature reported this finding in bGH mice of younger ages (Table 1 in background chapter) and few studies looked beyond 8 months. From this study, it appeared that hyperinsulinemia occurs only in young mice (perhaps due to a greater fat mass of bGH than WT when young), normalizes at middle ages (bGH started having less fat than WT mice at 6 months), and develops into hypoinsulinemia at older ages (much less fat). The reason for this phenomenon is not known. In addition to the fat mass theory above, another
possibility is that beta cells in young bGH mice are stimulated by high levels of GH and produce high amounts of insulin, as has been shown previously (A. Bartke et al., 2004; Fernando P. Dominici et al., 1999a; F. P. Dominici et al., 1999b; Olsson et al., 2003; Olsson et al., 2005; Zhihui Wang et al., 2007); however, chronic exposure to high GH leads to accelerated aging and this ‘aging’ phenotype may be manifested in multiple organs, including pancreas, which may fail to secret insulin as bGH mice get old. In this regard, GH/IGF-1 is known to stimulate beta cell proliferation and insulin secretion (Nielsen et al., 2001; Okuda et al., 2001; Parsons et al., 1995; Rhodes, 2000). However, no data to date have demonstrated an aging effect on pancreatic beta cells in bGH mice. Although it was reported that bGH mice as old as 8 months still had higher insulin levels than controls (Frick et al., 2001a), there was also a report that showed non-significantly different insulin levels (only showing an increased trend) compared to controls at 5 months of age (Berryman et al., 2006).

Normal glycemia was maintained in WT mice during aging, but decreased in bGH mice such that they had normal glucose up to 9 months but significantly lower levels in 12, 16 and 19 months. Similarly, lower than control glucose levels, although sometimes not significant, were observed in 3-month old hGH mice (A. Bartke et al., 2004), 11-month old (Rocha et al., 2007) and 8-month old bGH mice (Frick et al., 2001b). It seems that hypoglycemia develops in bGH mice at older ages. It is interesting that after 9 months, both fasting insulin and glucose dropped as a function of age and the levels were lower than those of WT mice. One possible explanation is that the prematurely ‘old’ bGH liver failed to secret normal amounts of glucose, since fasting blood glucose is mainly
derived from hepatic output (Nordlie et al., 1999; Sheehan, 2004). In support of this, a transcriptional coactivator regulating hepatic glucose production, PGC-1, which normally is induced by fasting in mouse liver to upregulate gluconeogenesis-related enzymes (Yoon et al., 2001), was lower in bGH than WT mice (Al-Regaiey et al., 2005). Conversely, PGC-1 was elevated in GHR−/− versus WT mice (Rocha et al., 2007). In addition, a key enzyme in gluconeogenesis, PEPCK, was lower in bGH mice in response to fasting (Valera et al., 1993). The levels of insulin, glucose, and body weight decline after 9 months of age, which again suggests that in bGH mice, 9 months may be a ‘definition’ of ‘old’. Additional support for this comes from data regarding cardiovascular health. In 8 or 9 month old bGH mice, cardiac function significantly decreases versus age-matched controls in terms of energy reserve, cardiocyte apoptosis and fatty acid metabolism (Bogazzi et al., 2009; Bogazzi et al., 2008; Bollano et al., 2000).

As mentioned in the introduction of this chapter and the background chapter I of this dissertation, bGH mice age prematurely and develop multiple organ pathology. The data on body composition, glucose and insulin data confirmed that bGH mice possess an accelerated aging process. Thus, it is possible that these age-related differences are reflected in changes in plasma proteins that could ultimately provide biomarkers of aging.

**Plasma proteins that were significantly different in bGH mice versus controls**

**ApoE is increased in bGH mice**

Five apoE isoforms have been identified (Figure 15 in chapter III). ApoE has a theoretical Mw of 34 kDa and the observed Mw on the gel for four of the isoforms (spots
4-7 in Figure 33) was ~34 kDa. An additional ApoE isoform (spot 2 in Figure 33) was ~38 kDa. The PTM for the size difference of this isoform is not clear. This additional isoform of 38kDa has not been reported previously. Although not reported in mice, in human plasma, apoE is glycosylated and glycated, which could in theory result in protein spots of slightly different Mw and also different pIs.

All five apoE isoforms increased significantly in bGH versus WT mice, with total apoE increasing 6 fold, indicating a positive effect of GH action. Consistent with this, hypophysectomized (Hx, removal of pituitary gland) female rats had decreased levels of apoE, but upon GH treatment, serum apoE levels nearly normalized (Frick et al., 2002; Jan Oscarsson et al., 1991b; Sjoberg et al., 1994a; Sjoberg et al., 1994b). This apoE increasing effect was not mediated by IGF-1 (Sjoberg et al., 1994b). GH has been shown to increase apoE by increasing the translation and secretion rate of apoE from hepatocytes (Sjoberg et al., 1994a). Continuous, but not pulsatile, treatment of GH increased apoE in Hx rats (J. Oscarsson et al., 1991a; Jan Oscarsson et al., 1991b). This suggested a sex-dimorphic effect of GH in regulating apoE expression, since males have a pulsatile secretion of GH during a 24 hour period whereas females have a more constant secretion pattern in mice, rats and humans (Leppaluoto et al., 1975; Norstedt & Palmiter, 1984; Saunders et al., 1976; Gloria Shaffer Tannenbaum & Martin, 1976). These reports are in agreement with the data presented in this study, since bGH mice have relatively high, constant levels of serum GH and should therefore upregulate production of ApoE. The novel data in this report are that different plasma isoforms of ApoE are all up regulated.
Several human studies have also confirmed GH’s effect on increasing apoE. Acromegalic patients had increased apoE levels in blood (Wildbrett et al., 1997). GH treatment in adult GH deficient patients for one year increased serum apoE levels (Johannsson et al., 1995). GH treatment of obese men showed an increase in apoE at 2 weeks although not 8 weeks (Svensson et al., 1999). However, in one study, GH treatment of 30 abdominally obese men for 9 months showed no change in serum apoE levels (Svensson et al., 2000). One possible reason is the dose difference of GH: bGH mice and acromegalic humans have much higher GH concentrations than that used for treatment of human subjects. Another likely reason is that the human subjects in these studies are obese, versus the lean mice used in this study.

ApoE is secreted primarily by the liver and functions to transport lipids via chylomicron, very-low-density lipoprotein (VLDL), LDL and high-density lipoproteins (HDL). VLDL is made by liver and transports lipids to other tissues to be used. Relatively high levels of VLDL are linked to increased risk of atherosclerosis (Veniant et al., 2008). LDL particles are formed from VLDL particles by the loss of triglyceride and gain of cholesterol ester. VLDL and LDL are considered ‘bad’ cholesterol because of a positive association with increased cardiovascular risks. HDL transports lipids from peripheral tissues back to the liver and is considered the ‘good’ cholesterol that is protective to cardiovascular health. ApoE-/- mice, deficient in apoE that transports and mediates the uptake of VLDL and LDL, have hypercholesterolemia and develop spontaneous atherosclerosis (Nakashima et al., 1994). Interestingly, GH exacerbated the atherosclerotic lesions in these mice even further, whether fed with chow or high fat diet.
bGH mice have increased plasma total cholesterol, including both HDL and LDL cholesterol (Frick et al., 2001a; Olsson et al., 2005). Perhaps increased apoE is made to transport the increased cholesterol.

It is interesting that apoE levels decreased in mice during aging. If GH stimulates apoE, then this is not surprising, since serum GH levels decrease with age. ApoE also is considered an antioxidant that helps to fight oxidative stress (Davignon, 2005; Miyata & Smith, 1996; Tarnus et al., 2009). Liver DNA damage increases with age in apoE−/− mice, but not in control mice (Folkmann et al., 2007). ApoE has also been shown to be coorelated with a downregulation of inflammation in the central nervous system in response to immune insult (Lynch et al., 2001). Since increased oxidative stress and reduced ability to fight it is one marker of aging (Dowling & Simmons, 2009), it appears that less apoE at old age reflects the reduced anti-oxidative capacity in the elderly. Contrary to the result in mice, apoE in human serum appears to increase with age (Schiele et al., 2000). This may be due to species difference.

**Hp is increased in bGH mice and shows a significant interaction between genotype and aging**

As discussed in chapter III, four Hp isoforms have been identified (see chapter III for Mw, pI and possible PTMs for Hp). All four Hp isoforms increased, with total Hp increasing 8 fold in bGH versus WT mice. Hp is an acute phase protein primarily secreted by liver. Acute phase proteins are induced in the liver by inflammation, infection or other immune insults. Hp has been found to increase in response to GH treatment in Turner’s syndrome (TS) patients (Gravholt et al., 2004). GH also increased Hp
production in a human hepatoma cell line (Derfalvi et al., 2000). In the mouse, Hp increased following whole-body exposure to gamma-rays (Rithidech et al., 2009). In pigs and lambs treated with acute insults (endotoxin and yeast), serum Hp as well as GH levels increased (L. G. Moore et al., 1995; Spurlock et al., 1998).

Since Hp is an indicator of inflammation, and that Hp increased during aging in both bGH and WT mice, it may indicate that mice had increased inflammatory state as they age. bGH mice had higher Hp levels than WT, and interestingly, two of the Hp isoforms showed an enhanced increase during aging in bGH relative to WT mice. This suggests that bGH mice may have an increased inflammation process during aging compared to WT mice.

**Clusterin showed significant interaction between genotype and aging**

We have identified 6 clusterin isoforms (Figure 15 in chapter III). The clusterin precursor is cleaved into α- and β-subunits, which are held together by five disulfide bonds. The heterodimer is extensively glycosylated with sulfated N-linked carbohydrates and secreted (Pajak & Orzechowski, 2006). Incidentally, the 6 isoforms observed on our 2D gels were clusterin β-chain, as indicated by MS/MS matching (Table 15 in appendix A). Clusterin β-chain has a theoretical Mw of 23.9 kDa and pI 6.12. The observed values on the gel were 34-36 kDa and pI 5.28, 5.37, 5.47, 5.59, 5.69 and 5.77. These are likely the result of PTMs such as glycosylation and phosphorylation. Clusterin is known to be glycosylated with sulphated carbohydrates, which could explain the increased Mw and a shift of pI to become more acidic. Phosphorylation may also contribute to this pI shift. According to an on-line tool to calculate Mw and pI with possible phosphorylation
modification at Swiss-Prot, ‘ScanSite pI/Mw’ (http://scansite.mit.edu/calc_mw_pi.html), clusterin β-chain would have pIs of 5.73, 5.57, 5.43 and 5.3 if it were to be phosphorylated on 1, 2, 3, or 4 residues (this tool has been applied to all proteins that have been identified in this dissertation; only certain clusterin and a2m isoforms (shown later) can be explained by phosphorylation). Interestingly, several of these numbers are very similar to the observed pIs. For example, with 1 phospho (P) group, the theoretical pI would be 5.73 and spot 20 (Figure 15 in chapter III) has a pI of ~5.77; with 2 P groups, theoretical pI 5.57 and spot 18 has a pI of ~5.59; with 3 P groups, the theoretical pI 5.43 and spot 17 is at ~5.47; and with 4 P groups, the theoretical pI would be 5.3 and spot 15 was at ~5.28. Therefore, it is very likely that the difference in pI of clusterin is due to differential levels of phosphorylation. It is curious that the clusterin α-chain, with a theoretical Mw 25 kDa and pI 5.09, was not observed on our gels. One possibility is that α-chain is sulphated and if also phosphorylated, then the pI will shift to a pI less than 5, which is beyond the pI range of the gel analysis performed in this work (pI 5-8). In a previous report (Duan et al., 2004), where a broader pI range was used for 2-DE, clusterin was identified as two distinct protein ‘spot’ trains, one with pIs of ~5-6, and the other with pI of ~4-5 with a slightly larger Mw. Although not specified in that paper, it is likely that the two trains corresponded to β- and α- chains, respectively (notably, MS/MS sequence coverage was 22.5% for the more acidic train and 31% for the other, implying that only a fragment of the precursor was matched). Similarly in a human plasma 2D gel database, clusterin is also resolved as two trains; one at a pI of ~4.7-5.1 and the other at a pI of ~5.2-5.8. Since clusterin is synthesized as a precursor containing both chains, the
quantity of β-chain should in theory represent that of the full-length clusterin. Therefore, the finding regarding clusterin β-chain should reflect that of total clusterin levels.

Clusterin did not change in WT mice with age, but increased significantly in bGH mice. Initially lower in bGH mice, clusterin increased to higher levels than WT at later ages. This was true for two isoforms as well as total level, with a similar trend in other isoforms, but not reaching p<0.01 of statistical significance. Total clusterin decreased significantly (p<0.01) in bGH versus WT mice, although not markedly (87% of WT); and this decrease was only because of the lower levels at 2 and 4 months of age. At older ages, clusterin was higher in bGH than WT mice.

Clusterin is expressed in several tissues, mainly in liver, brain and stomach. It is a molecule involved in a wide range of biological processes, and surprisingly has several names, such as sulfated glycoprotein 2 and apolipoprotein-J. Clusterin binds to cells, membranes and hydrophobic proteins for scavenging abnormally aggregated or denatured proteins and is also involved in apoptosis. It is an apolipoprotein that associates with HDL. Interestingly, clusterin is found to accumulate in atherosclerotic plaques (Jordan-Starck et al., 1994; Witte et al., 1993). To our knowledge, no report to date mentioned a relationship between GH and clusterin. Thus, changes in clusterin isoforms may be a marker of GH action, specifically, spots 28 and 29 that significantly increased in bGH mice at older ages.

More data concerning clusterin and aging is presented below. In humans at autopsy, it was found that the pituitary content of clusterin increased with age, suggesting a role of clusterin in the aging of the pituitary (Ishikawa et al., 2006). Also, secreted clusterin was
higher in the kidney samples of patients with renal cell carcinoma than healthy subjects (Kurahashi et al., 2005; Miyake et al., 2002). In mice, clusterin is expressed in the kidney during embryonic development but diminishes subsequently (French et al., 1993; Harding et al., 1991). However, clusterin is induced in kidney under certain injury/disease conditions such as ischemic renal injury, acute kidney injury by ablation and glomerular hypertension (Correa-Rotter et al., 1992; M. E. Rosenberg & Paller, 1991; Singhal et al., 1998). In fact, clusterin is used as a marker for severity of kidney tubular damage in rats (Hidaka et al., 2002). This is very interesting because bGH mice are known to develop progressive glomerulosclerosis as they age (Quaife et al., 1989) and glomerulosclerosis is detected as early as 7 months (T. Doi et al., 1990). Note that clusterin (spots 28, 29 and total) in bGH mice started to surpass controls at 8 months (Figure 45 and Figure 46).

Additional data on clusterin and kidney damage has been reported. Young clusterin -/- mice have normal kidneys; however, 21-month old knockout mice develop glomerulopathy (Mark E. Rosenberg et al., 2002). In humans, lower levels of plasma and urine clusterin are associated with renal diseases (Ghiggeri et al., 2002). These studies suggest a protective role of clusterin against renal disease. Therefore, it is possible that bGH mice secret higher levels of clusterin as a protective mechanism for the progressive kidney disease.

It is possible that the higher level of clusterin noted in this study were secreted from the damaged kidneys of bGH mice at 8 months and older. If this possibility could be confirmed, then clusterin could be a marker of excess-GH-induced kidney damage, either
directly by GH or indirectly from kidney damage via a yet unknown mechanism. However, bGH mice had lower than normal clusterin in plasma at 2 and 4 months, suggesting that at young ages, GH suppressed clusterin expression. Clusterin thus could be a very important factor when studying age-related diseases in bGH mice, because it is suppressed in young bGH mice, but is increased dramatically in bGH mice as a function of age and eventually surpassing levels in WT mice.

One isoform of MBP-C is increased in bGH mice

Three isoforms have been identified as MBP-C (Figure 15 in chapter III). MBP-C has a theoretical Mw 24 kDa and pI ~5. PTMs of MBP-C include hydroxylation on proline residues and N-linked glycosylation. The three isoforms identified on 2D gel were ~32 kDa, possibly could be explained by glycosylation and proline oxidation. The isoform reported in this paper increased in bGH mice. The other two isoforms were also higher in bGH mice. However, due to large variability between individual mice, they were not reported here. MBP-C, also called mannan-binding lectin (MBL), is primarily synthesized by the liver and is also an acute phase protein. It binds mannose and N-acetylglucosamine in a calcium-dependent manner. It is an innate immune defense protein that is involved in defense against pathogens by activating the classical complement pathway independently of antibodies (Turner, 2003). In the serum, MBP-C is composed of 18 subunits (6 units of homotrimers) (Kurata et al., 1994). Different human alleles of MBP-C have been identified that affect immune functions, e.g., genetic variations in MBP-C are associated with susceptibility to hepatitis B virus infection.
(HBV infection) (Thio et al., 2005). Also, low levels of MBP-C were associated with susceptibility to frequent and chronic infections (Sumiya et al., 1991; Turner, 2003).

GH has been linked to modulation of innate immunity (T. K. Hansen, 2003; Kelley et al., 2007). GH treatment of Turner’s syndrome patients increases MBP-C level in plasma (Gravholt et al., 2004). GHD patients have lower and acromegalic patients have higher baseline MBP-C levels than healthy subjects. Also, when treated with GH, healthy subjects have 2-fold increase and GHD patients 4-fold increase in MBP-C, whereas acromegalic patients treated with octreotide or pegvisomant had reduced MBP-C by 33% (T. K. Hansen et al., 2001). This MBP-C increasing effect of GH was independent of IGF-1 as IGF-1 treatment did not change MBP-C levels, both in the serum and in cultured hepatocytes (C. M. Sørensen, 2006; T. K. Hansen et al., 2001).

In addition to innate immunity, GH also stimulates acquired immunity (Blazar et al., 1995; Dialynas et al., 1999; Postel-Vinay et al., 1997). GH is thought to have a positive action on T cell development, cytokine production, B cell development, antibody production and priming neutrophils and monocytes for superoxide anion secretion (Hattori, 2009). However, controversy exists and bGH mice do not appear to have an immune system that better combats infection. On the contrary, they produce less specific antibodies when challenged with tetanus toxoid while GH deficient Ames dwarf mice produce normal levels of antibody (Hall et al., 2002). Thus the complete role of GH in immune function is still not clear. However, the increase of MBP-C observed in bGH mice may be a good indicator of GH action in innate immunity.
TTR decreased in bGH mice

As discussed in chapter III, 9 TTR isoforms have been identified in 2D gels. Eight of these isoforms decreased in bGH compared to WT mice at all ages examined. Interestingly, it has been reported that serum TTR is decreased by GH treatment in Hx male rats of 2 months of age (Vranckx et al., 1994).

TTR is primarily secreted by the liver. It forms a homotetramer that transports thyroxine (T4) and RBP-4. TTR is also synthesized by retinal pigment epithelium (RPE), choroid plexus (Ong et al., 1994; Schreiber, 2002) and pancreas (Itoh et al., 1992; Jacobsson et al., 1990). TTR synthesized and secreted by choroid plexus is the major protein in the cerebrospinal fluid (CSF) and is involved in the transport of T4 across blood brain barrier (Schussler, 2000). TTR also regulates β-cell function. In cell cultures, TTR promoted insulin secretion upon glucose stimulation and protected pancreatic β-cells from apoptosis (Refai et al., 2005).

TTR and RBP-4 were found to be decreased in type I diabetic patients and type I diabetic rats (Itoh et al., 1992; Refai et al., 2005; Tuitoek et al., 1996). TTR is also an acute phase protein that is suppressed during inflammation, infection or malnutrition (Fleck, 1989; Ingenbleek & Young, 1994; Lasztity et al., 2002; Seres, 2005). The lower levels of TTR are associated with higher rate of morbidity/mortality and recovery from acute and chronic disease (Fuhrman et al., 2004). It is possible that decreased TTR in bGH mice indicated an increased inflammation state in these giant mice. In fact, TTR is a negative acute phase protein that is suppressed by inflammation (Fleck, 1989). Therefore the lower levels of TTR in bGH mice indicate an inflammatory state in these mice.
Interestingly, total TTR levels in the plasma correlated with those of RBP-4 in WT mice, and to a less extend, in bGH mice (Figure 40 and Figure 42A), which suggests retention of RBP-4 in the plasma from kidney clearance by TTR. Also, TTR increased during aging in both WT and bGH mice (Figure 40). In particular, as shown in chapter III, one isoform of TTR was a positive biomarker of aging. Together, TTR is suppressed by GH but increased by aging.

**ApoA1 decreased in bGH mice**

Six apoA1 isoforms have been identified (Figure 15 in chapter III). The theoretical Mw and pl of apoA1 are 28 kDa and 5.4, respectively. Observed values of these isoforms on our gels were 28 kDa and pl ranging from 5.73-6.4. Of these, spots 17-19 decreased in bGH mice.

ApoA1, synthesized by liver and intestine, is the major protein component in HDL, which is thought to have antiatherogenic and anti-aging effects (Nofer et al., 2005). Plasma concentration of apoA1 is closely associated with HDL cholesterol, and upregulation of endogenous apoA1 level, either by increasing production or by decreasing clearance, is considered to be a promising approach to improve cholesterol generated cardiovascular disease (Santos-Gallego et al., 2008).

Acromegalic patients had increased serum apoA1 levels after treatment with somatostatin analogues (Sideris P. Delaroudis, 2008). However, several studies have shown decreased apoA1 in GHD patients or Hx rats and increased apoA1 when treated with GH (Cenci et al., 2008; Cuneo et al., 1993; Jan Oscarsson et al., 1991b; Svensson et al., 2000). Thus, too much and too little GH both reduce apoA1. bGH mice mimic
acromegaly in humans, and the total apoA1 in bGH mice indeed was decreased (p=0.011). Importantly, three isoforms decreased with p<0.01, with others not changing significantly. This suggested that over-expression of GH decreased apoA1 plasma levels, possibly affecting the PTMs, and therefore the composition of apoA1 isoforms in plasma. It is not clear how this is regulated, but may be relevant to cardiovascular disease propensity when GH is over-expressed.

**RBP-4 levels decrease as well as isoform-specific changes in bGH mice**

RBP is in theory 21.6 kDa with a pI of 6. We observed 7 isoforms, with isoforms 1-3 being 22 kDa and isoforms 4-7 being 19 kDa, and a wide range of pI from 5.28 to 7.61 (Figure 15). It appeared that the 22 kDa isoforms were the full length, and the 19 kDa isoforms may be a fragment. The different pIs of the 19 kDa RBP-4 isoforms are likely the result of phosphorylation (unpublished observation by Okada).

RBP-4 is mainly secreted by liver and adipose tissue. RBP-4 binds to retinol and delivers it from liver to peripheral tissues. In blood, RBP-4 is bound to TTR homotetramer, which increases its half life by reducing kidney filtration. RBP-4 has been associated with obesity and insulin resistance (Haider et al., 2007; Kowalska et al., 2008; Kohzo Takebayashi et al., 2007a) and has been found to increase in type II diabetic subjects (Raila et al., 2007).

Since RBP-4 is associated with obesity, it is not surprising that total RBP-4 in bGH mice was lower than WT, as bGH mice had much less fat than WT mice. Similar results have been reported. For example, serum RBP-4 was decreased in GHD children after 6 weeks of GH treatment (Kemp & Canfield, 1983). In Hx rats, vitamin A (a retinol that is
bound by RBP-4) was cleared from blood much slower than controls, and a single GH injection decreased vitamin A in plasma and increased its level in the urine significantly (Ahluwalia et al., 1980). These results suggest that GH promotes vitamin A clearance, possibly by clearance of RBP-4 and thus decreasing its levels in plasma. Since GH also decreases TTR, GH may reduce RBP-4 through reducing TTR. Interestingly, like TTR, RBP-4 is also considered a negative acute phase protein (Fleck, 1989), whose decreased levels indicate inflammation. Thus, by decreasing both TTR and RBP-4, excess GH may promote inflammation in bGH mice.

In addition to total RBP-4 reduction, RBP-4 also showed isoform-specific changes. For example, despite overall decrease and in particular spot 21, spot 9 actually increased. Notice that these two isoforms were of different sizes. Spot 9 was 19kDa and spot 21 was 22kDa (Figure 33). This indicated that GH favored the accumulation of 19 kDa rather than 22 kDa RBP-4. The nature of the PTM responsible for the size difference may therefore be regulated by GH. For example, GH may upregulate a proteolytic enzyme that cleaves the full-length RBP-4 to give rise to the 19kDa fragment.

**A2m showed isoform-specific changes in the plasma of bGH mice**

Seven isoforms have been identified as a2m (Figure 15 in chapter III and appendix A). In mice and rats, a2m is composed of two subunits- 165 kDa and 35 kDa, presumably through intracellular proteolytic processing of a precursor protein by an yet to be defined proteinase (van Leuven et al., 1992). In this study, the 35 kDa subunit was detected (Figure 33), and MS/MS also matched peptides in this subunit (Table 15 and Figure 84 in appendix A). The 165kDa subunit was beyond the range of our gels and therefore was
not detected; however, since the two subunits are held together by disulfide bond, and are disrupted during the 2-DE process, this 35kDa subunit should represent the presence of the intact protein. The 35kDa subunit of a2m has a theoretical pI of 7.09 and the seven observed isoforms had pI ranging from 5.65 to 7.08, which means isoform 7 (refer to Figure 15) is most likely the un-modified form and isoforms 1-6 have been modified by PTM(s). A2m is phosphorylated (Villen et al., 2007) and glycosylated on multiple Asn residues in both subunits (Ghesquiere et al., 2006). These PTMs are likely the reason for a2m to exist as multiple isoforms with similar sizes but different pIs on the 2D gel. Using the above-mentioned tool to calculate Mw and pI with possible phosphorylation modification (http://scansite.mit.edu/calc_mw_pi.html), it was revealed that isoform 2 (observed pI 5.85) was consistent with addition of three phosphate groups (predicted pI 5.81) and isoform 3 (observed pI 5.08) was consistent with addition of two phosphate groups (predicted pI 5.08). Other isoforms have observed pIs not explained by phosphorylation alone, and may be subjected to glycosylation and/or a combination of phosphorylation and glycosylation.

Interestingly, although total a2m did not change, four of these isoforms showed differential regulation by GH. Spot 1 and 3 increased whereas spots 15 and 16 decreased in bGH mice, suggesting that GH regulated the PTMs of plasma a2m.

A2m is a plasma proteinase inhibitor that is mainly secreted by liver (Lorent et al., 1994). It is also expressed in ovary, heart, stomach, kidney, lung and uterus (He et al., 2005). It binds to all four classes of proteases (including aspartic, métallo, cysteine, and serine proteases) to form a complex, which is cleared via receptor-mediated endocytosis.
Both native (free) and conformationally transformed (when binding to a protease) α2m can bind a variety of cytokines, growth factors and hormones, and is thought to play roles in hormonal control, immune modulation, signal transduction, growth and differentiation (Tayade et al., 2005). Interestingly, in plasma, the conformationally transformed α2m binds GH, although with weaker affinity than GH binding protein (BP) (Adham et al., 1969; Kratzsch et al., 1995, 1996). A2m also binds to IGFBP-1 and modifies IGF-I/IGFBP-1 actions resulting in enhanced IGF effects (Westwood et al., 2001). A2m is an acute phase protein induced by inflammation (de Boer et al., 1993; Fleck, 1989). A knockout mouse model deficient in α2m were normal under standard conditions but had an increased mortality rate and more severe symptoms when subjected to acute pancreatitis (Umans et al., 1999).

GH increased α2m production in a human hepatoma cell line (Derfalvi et al., 2000). Acromegalic patients had increased α2m levels (Pozzilli et al., 1978). Patients with somatotrophic adenoma had elevated levels of α2m in blood, but normalized one year after Hx, together with reduced serum GH levels (Zargarova & Konnova, 1983). A2m is induced by prolactin in rat ovarian granulosa cells (Dajee et al., 1996) and decidual cells (Barkai et al., 2000) through the Jak2-Stat5 pathway. Although it has not been reported, it is possible that GH induces α2m in the liver through a similar mechanism. My results indicate a novel regulation of GH on α2m through PTM, rather than at the total level change.
Conclusion

2-DE of the plasma of bGH and WT mice at different ages revealed that several proteins increased (apoE, Hp and MBP-C) or decreased (TTR, RBP-4 and apoA1).

- All five apoE isoforms identified increased in bGH mice compared to WT mice at all ages, resulting in a total increase of 6 fold.
- All four Hp isoforms increased in bGH mice compared to WT mice, resulting in a total increase of more than 8 fold.
- Out of three isoforms of MBP-C, one increased more than 8 fold in bGH compared to WT mice.

The above isoforms and/or proteins are positive biomarkers of GH.

- Out of nine isoforms of TTR, eight decreased at all ages, resulting in a 60% reduction of total TTR in bGH compared to WT mice.
- Out of six apoA1 isoforms, three decreased, ranging from 46-66% reduction compared to WT levels. Moreover, total apoA1 decreased in bGH mice (p=0.011). Thus, apoA1, in particular these three isoforms, are negative biomarkers of GH.
- One isoform of RBP-4 (spot 9) increased 3 fold in bGH mice; however, another isoform (spot 21) decreased 40%. The total RBP-4 is reduced 25% in bGH mice compared to WT mice. Therefore, total RBP-4 as well as spot 21 are negative biomarkers of GH, but spot 9 is a positive marker.
- Similarly, out of the seven a2m isoforms, two increased (spots 1 and 3, four and two fold, respectively) and two decreased (spots 15 and 16, 26% and
57% reduction, respectively). Thus, spots 1 and 3 of a2m are positive markers and spots 15 and 16 are negative markers of GH.

Of particular interest is that Hp and clusterin exhibited significant interaction between GH and aging. All four Hp isoforms increased more vibrantly in bGH mice during aging than in WT mice. Two isoforms of clusterin as well as total clusterin increased during aging to a higher extent in bGH mice versus controls. Therefore, Hp and clusterin are possibly related to the accelerated aging in bGH mice.

Many of the proteins are secreted by liver as acute phase proteins (proteins produced by liver to promote inflammation, activate the complement cascade, and stimulate chemotaxis of phagocytes in response of acute infection, injury and inflammation) either induced (Hp, MBP-C, a2m) or suppressed (TTR, RBP-4) by acute inflammation, infection or other immune insults. One common emerging theme is that in the bGH mice, ‘positive’ (protein that is induced by inflammation) acute phase proteins (Hp, MBP-C) increase, but ‘negative’ ones (protein that is suppressed by inflammation, such as TTR and RBP-4) decrease, suggesting an increased inflammatory state in bGH mice. In fact, many acute phase proteins are induced by cytokines through Jak-Stat signaling pathways. GH signals through a Jak2-Stat5 pathway (Frank et al., 1994; Kopchick & Andry, 2000; Lanning & Carter-Su, 2006; Waters et al., 2006). Acute phase proteins are a part of innate immune system and are important players in combating acute infection and injury. However, the chronic upregulation of these proteins in the absence of an ‘acute infection/injury’ in bGH mice reflect a inflammation-prone phenotype. This could explain partially the shortened lifespan of bGH mice, since increased inflammation is
associated with aging (Brüünsgaard & Pedersen, 2003; Ferrucci et al., 2005; Roubenoff et al., 1998).

Another theme appears to be that GH increases VLDL and LDL-associated lipoprotein (apoE), but decreased HDL-associated lipoprotein (apoA1 and clusterin). It is known that HDL is the ‘good’ cholesterol and beneficial to cardiovascular system and VLDL and LDL are ‘bad’ and increase cardiovascular disease risks. This could also contribute to reduced lifespan of bGH mice. However, bGH mice are reported to have increased HDL and LDL, and decreased VLDL at 6 months (Frick et al., 2001a). Since apoE is also an apolipoprotein for HDL, it is possible that the increased HDL cholesterol in bGH mice is carried by apoE, compensating for reduced apoA1 and clusterin which are carriers of HDL. This suggests that bGH mice not only have altered lipid profiles, but also altered apolipoproteins associated with HDL and LDL. The significance of this needs further study.

In conclusion, several plasma proteins have been identified that are up- or downregulated in bGH mice. These proteins and their specific isoforms are biomarkers of chronic GH action. Some proteins show isoform-specific changes, thus it will be important to identify PTMs corresponding to these isoforms. These biomarkers of GH indicate an increased inflammatory state and an altered lipid profile in bGH mice.
Abstract

Growth hormone receptor gene knockout (GHR KO or GHR-/-) mice are dwarf, insulin sensitive and long-lived despite having an obese phenotype. We hypothesize that the plasma protein profiles of these mice in comparison with wild type (WT) control mice would possess novel biomarkers for the lack of GH action and may also be correlated with longevity. In this paper, we have analyzed plasma proteins of GHR-/- and WT mice of both genders using two-dimensional electrophoresis (2-DE) and identified several plasma markers for the lack of GH action. GHR-/- mice had increased apolipoprotein A-4 (apoA4), decreased apoE and isoform-specific changes in retinol-binding protein-4 (RBP-4) and apoA1. Interestingly, certain proteins showed gender difference. Female mice had increased isoform(s) of apoE and RBP-4; decreased apoA1, clusterin, Hp and hemoglobin beta chain; and isoform-specific changes in albumin. A significant interaction between gender and aging was found in the levels of isoform(s) of apoA4, apoA1, transthyretin, albumin and RBP-4. Specific isoform(s) of apoE, RBP-4 and Hp showed a significant interaction between gender and genotype. Together, these data demonstrated marked difference between GHR-/- and WT mice, as well as a gender specific difference in plasma proteins.
Introduction

Growth hormone receptor/binding protein gene knockout (GHR KO or GHR-/-) mice are dwarf and have markedly reduced serum IGF-1 and elevated GH levels. They are obese, yet insulin sensitive and long-lived (Berryman et al., 2004; Coschigano et al., 2000; Coschigano et al., 2003; Liu et al., 2004). Many studies on GHR-/- mice show phenotypic differences including their increased adipose tissue mass, particularly in the subcutaneous depot (Berryman et al., 2004; Berryman et al., 2006; Liu et al., 2004). These mice also have reduced muscle fiber size (Schuenke et al., 2008; Sotiropoulos et al., 2006) and reduced bone length and bone mineral density (Sjogren et al., 2000). GHR-/- mice have a disproportionally enlarged brain compared to WT animals (Asa et al., 2000; Ransome et al., 2004) and have less age-associated memory loss that is seen in wild type (WT) mice (Kinney et al., 2001). Additionally, GHR-/- mice have delayed sex maturation and are sub-fertile (A. Bartke, 2000; Chandrashekar et al., 2001a; Chandrashekar et al., 2001b; Keene et al., 2002; Zhou et al., 1997). Despite a smaller heart phenotype, GHR-/- mice have normal cardiovascular function (E. Egecioglu et al., 2007; Izzard et al., 2009). GHR-/- mice are resistant to streptozotocin-induced kidney damage (Bellush et al., 2000), in contrast to bGH mice, which progressively develop glomerulosclerosis (T. Doi et al., 1990; T. Doi et al., 1988; Quaife et al., 1989). Finally, GHR-/- mice have decreased cancer incidence, including breast, lung, pituitary, lymph and prostate (Ikeno et al., 2009; Kineman et al., 2001; Z. Wang et al., 2005; X. Zhang et al., 2007). The longevity seen in this mouse model is thought to be at least in part
overlapping with life extension in caloric restricted animals (Bonkowski et al., 2009; R. A. Miller et al., 2002).

Despite much research on the GHR-/- mice, little has been done at the proteomic level with even less associated with the life span of these mice. Proteomics explores the entire set of proteins in a given tissue under a certain condition. Novel proteins that are differentially regulated in GHR-/- mice versus WT will provide insight into the long-lived phenotype of this mouse model. Specifically, we hope to identify plasma proteins that serve as potential markers of the lack of GH action and increased longevity. In this longitudinal study, plasma proteins were analyzed in both male and female GHR-/- mice of adult (8 months), middle age (16 months) and old (24 months) relative to WT controls using a two-dimensional gel electrophoresis (2-DE). Proteins differently expressed in the plasma were identified by mass spectrometry (MS) and tandem MS (MS/MS).
Materials and Methods

**Experimental animals**

The GHR-/- mouse line was described previously (Zhou et al., 1997). The mice were originally generated in the background of 129Ola/BalbC. They were subsequently backcrossed with C57BL/6J mice for at least eight generations. GHR-/- mice of both genders (n=6 for both male and female) and their wild type (WT, or GHR+/+) littermate controls (n=5) were analyzed at the following ages: 8, 16 and 24 months. These ages represent young-middle (8 months), middle (16 months) and old age (24 months) in mice (Richard A. Miller & Nadon, 2000). Mice were housed 2-3 per cage at room temperature (22 °C) in a 12-hour light, 12-hour dark cycle. Mice were fed ad lib with chow diet. Animal protocols were approved by Ohio University’s Institutional Animal Care and Use Committee.

**Body composition measurement**

Body composition of 12-month old mice was measured using mq 7.5 NMR analyzer the Bruker Minispec (The Woodlands, TX) as described previously (List et al., 2009; Palmer et al., 2009). Each animal was measured twice and the mean value used.

**Plasma IGF-1 measurement**

Blood was collected with heparinized capillary tubes by tail tip clipping following heat lamp exposure. Whole blood was centrifuged at 7000 x g for 10min at 4 °C and the resulting plasma was stored at -80 °C. Plasma IGF-1 levels were determined using the DSL-10-29200 mouse/rat IGF-1 ELISA kit by Diagnostic Systems Laboratories, Inc. (Webster, TX).
**Fasting glucose and insulin measurements**

Mice were fasted for 4 hours and bled at 3 PM. Blood glucose was measured by ONE TOUCH glucometer from Lifescan (Milpits, CA). Plasma was collected as described above and insulin levels were measured by an ultrasensitive rat/mouse insulin ELISA kit (ALPCO, Windham, NH).

**2-DE and quantification of proteins**

See chapter III.

**Statistical analysis**

All statistical analyses were performed using SPSS 14.0 software (Chicago, IL). Data for IGF-1 was subjected to independent t test with p<0.05 as significant; data for body composition, fasting glucose and insulin were subjected to 2-way ANOVA test with gender and genotype as two factors (p<0.05). Data for body weight were subjected to repeated measures with significance value p<0.05. For protein intensity, the log-transformed data were subjected to repeated measures of three ages to examine an aging effect, with genotype and gender as two fixed factors to examine the effects of GH and sex (p<0.01). All data are presented as mean ± SEM.

**Protein identification by mass spectrometry (MS), MS/MS, and liquid chromatography (LC)-MS/MS**

See chapter III.
Results

Body weight and body composition

As mice aged from middle age (9 months) to old age (24 months), they showed significant weight change (two-way repeated measures, p<0.001, Figure 47 A). All animals gained weight from 9 to 16 months, and then showed a slight weight loss. GHR-/− mice had significantly reduced body weight than WT mice (p<0.001). There was no significant gender difference using repeated measures. However, if each age was viewed separately with 2-way ANOVA, female WT had reduced weights relative to male WT mice at 9 (p<0.01) and 12 months of age (p<0.05). Interestingly, GHR-/− had a smaller gender difference than WT mice. Also, female mice (both WT and GHR-/−) were less susceptible to weight loss at 24 months than males.

Figure 47. Body weight from 9 to 24 months and body composition at 12 months. A, body weight of mice of different ages. Two-way repeated measures showed a significant
effect of aging (p<0.001), genotype (p<0.001) and interaction between aging and
 genotype (p<0.05). B, body composition at 12 months in absolute mass. C, stacking bar
 graph of mean body composition in each group at 12 months. D, normalized lean mass
 (percentage of body weight) at 12 months. E, normalized fat mass (percentage of body
 weight) at 12 months. F, normalized fluid mass (percentage of body weight) at 12
 months. Different letters denote significant difference (p<0.05).

Body composition was measured at 12 months of age (middle age). The absolute
 mass of all body composition components was reduced in GHR-/- compared to WT mice
 (p<0.001 for body weight, lean and fluid mass, p=0.002 for fat mass, Figure 47 B). There
 was no gender difference in body composition in GHR-/- mice. In contrast, female WT
 had a reduced body weight (p=0.033) and lean mass (p=0.001) relative to male WT mice.
 Male and female WT mice had similar fat and fluid masses (Figure 47B). From Figure
 47 C, it is clear that the weight difference was entirely due to reduced lean mass in female
 WT mice, whereas fat and fluid mass were similar in both genders. Similarly, normalized
 lean mass (percentage of body weight) was significantly lower in GHR-/- compared to
 WT (p<0.01) without a gender effect (Figure 47 D); whereas normalized fat mass was
 significantly higher in GHR-/- mice (p<0.01) without a gender effect (Figure 47 E).
 Normalized fluid mass, on the other hand, exhibited a significant interaction between
 gender and genotype (p<0.01), being higher in WT female than WT male, but the same in
 GHR-/- females and males (Figure 47 F). Together, GHR-/- mice had disproportionally
 increased fat and decreased lean mass with no change in fluid mass compared to WT
 mice. There was no gender difference in GHR-/- mice, whereas in WT mice, females had
 reduced lean mass resulting in reduced total body weight.
IGF-1, fasting glucose and insulin levels

IGF-1 levels were determined for male mice at 6 months of age (Figure 48 B). IGF-1 was significantly lower in GHR-/- than WT mice (p<0.0001) as has been reported previously (Coschigano et al., 2000; Coschigano et al., 2003). Two-way ANOVA was used to test for the effects of gender and genotype on 4h-fasting glucose and insulin levels at two ages: 9 and 24 months. At 9 months, GHR-/- mice had significantly lower fasting glucose levels than WT controls (p<0.05), and females had significantly higher levels than males (p<0.05). However, no significant difference was found among the four groups at 24 months (Figure 48A). Fasting insulin levels, on the other hand, exhibited interesting features. At both ages, insulin was significantly lower in GHR-/- mice than WT controls (p<0.001). At 9 months, insulin was significantly lower in females than males in both genotypes (p<0.01), but not significantly different between genders at 24 months (Figure 48A). These values are similar to those reported previously (Al-Regaiey et al., 2005; Coschigano et al., 2003).

Together, these data suggest that GHR-/- mice (both male and female) have lower insulin levels than their WT counterparts at adult and old age, but have lower glucose levels than WT only at 9 months of age. Regarding gender difference, females have lower insulin levels than males at an old age, but not at a younger age. There is no gender difference in glucose levels.
Figure 48. IGF-1 and fasting glucose and insulin levels. A, 4-hour fasting glucose and insulin levels at 9 and 24 months. Different letters denote significant difference (p<0.05). B, IGF-1 levels of male mice at 6 months. *p<0.0001.

Plasma proteins that exhibited significant gender differences

Plasma proteins that showed changes in genotype, gender and aging revealed by two-way repeated measures were labeled 1-29 on a typical 2-D gel as shown in Figure 49. Proteins with significant gender difference are listed in Table 8 and those different by genotype listed in Table 9.
Figure 49. 2D gel map of identified proteins of interest. Proteins are marked by ‘x’ and numbered 1-31. Abbreviations: pI: isoelectric focusing point; Mw: molecular weight.

Table 8. Proteins significantly different in males and females (p<0.01)

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein ID</th>
<th>Isoform #</th>
<th>Ratio (F/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>apoE</td>
<td>1</td>
<td>↑3.2</td>
</tr>
<tr>
<td>16</td>
<td>RBP-4</td>
<td>7</td>
<td>↑2.4</td>
</tr>
<tr>
<td>Total</td>
<td>RBP-4</td>
<td>Sum</td>
<td>↑1.2</td>
</tr>
<tr>
<td>5</td>
<td>Clu</td>
<td>3</td>
<td>↓0.27</td>
</tr>
<tr>
<td>14</td>
<td>RBP-4</td>
<td>3</td>
<td>↓0.31</td>
</tr>
<tr>
<td>22</td>
<td>Hp</td>
<td>2</td>
<td>↓0.08</td>
</tr>
<tr>
<td>25</td>
<td>Alb</td>
<td>19</td>
<td>↓0.25</td>
</tr>
<tr>
<td>26</td>
<td>Hbb</td>
<td>1</td>
<td>↓0.11</td>
</tr>
<tr>
<td>27</td>
<td>Hbb</td>
<td>3</td>
<td>↓0.05</td>
</tr>
<tr>
<td>Total</td>
<td>apoA1</td>
<td>Sum</td>
<td>↓0.85</td>
</tr>
</tbody>
</table>

Spot # corresponds to Figure 49; isoform # corresponds to Figure 15 in chapter III. The ratio F/M was calculated as the mean value of female/mean value of male (all samples regardless of genotype). ‘↑’ means up-regulation and ‘↓’ means down-regulation.

Spots 4 (apolipoportein-E or apoE isoform) and 16 (retinol-binding protein-4 or RBP-4 isoform) were significantly higher in female compared to male mice regardless of
genotype \( (p<0.01, \text{Figure 49 and Table 8}) \). Since no genotypic difference was found for these proteins, the reported quantity ratio of female (F)/male (M) was calculated from pooled values of both genotypes. The same applies to Table 9, where no significant gender difference was found for a given protein, the data from both genders were pooled together. Spot 4 increased 3 fold and spot 16 increased 2 fold in females versus males. Total levels of RBP-4 (sum of all identified RBP-4 isoforms) also increased significantly \( (p<0.01) \) in females, although only at 1.2 fold.

Certain isoform(s) of clusterin (Clu), RBP-4, albumin (Alb), haptoglobin (Hp) and hemoglobin \( \beta \)-chain (Hbb) decreased significantly in females \( (p<0.01, \text{Table 8}) \). Total apoA1 also decreased significantly \( (p<0.01) \), although no individual spot of apoA1 showed significant decrease or increase. This may be due to an accumulative effect when adding up all the individual isoforms.

In addition, 14 proteins showed significant interaction between gender and aging \( (p<0.01, \text{Figure 50}) \), including two isoforms of apoA4, 4 isoforms of apoA1, one isoform of transthyretin (TTR) and RBP-4, as well as 6 isoforms of Alb.
Figure 50. Plasma proteins that exhibited significant differential change during aging between males and females (p<0.01). Protein # corresponds to Figure 49. A, proteins that increased during female aging but did not change in male aging. B, proteins that decreased during female aging but did not change in male aging.
Spots 1 and 2, two apoA4 isoforms, increased during aging in females with no aging difference in males (Figure 50, Figure 51 and Figure 52). Spots 8-11, four apoA1 isoforms, increased in female aging but did not show significant change in male aging (Figure 50 and Figure 53). Spot 17, a TTR isoform, increased in female aging to a larger degree than in male aging (Figure 50 and Figure 54). Spot 14, a RBP-4 isoform, decreased in female aging but increased at 16 months then decreased at 24 months in male aging (Figure 50 and Figure 55). Two albumin spots, 28 and 29, increased in female aging without change in male aging (Figure 50). However, four other albumin spots decreased in female aging with no change in male aging (Figure 50; also see Figure 54, Figure 56 and Figure 57).

Plasma proteins that exhibited significant genotypic differences

Four proteins showed a significant increase and three showed a significant decrease in GHR-/- compared to WT controls (p<0.001, Table 9). Two isoforms (spots 2 and 3) as well as total of apoA4 were upregulated in GHR-/- mice (Figure 51 and Figure 52). Two isoforms of RBP-4 (spots 13 and 14) were also upregulated in GHR-/- mice (Figure 55 and Figure 58). Another isoform of RBP-4 (spot 15), however, decreased in GHR-/- mice (Figure 56). One isoform of apoA1 (spot 8, Figure 53), as well as three isoforms (spots 4, 6 and 7, Figure 51 and Figure 52) and total levels of apoE decreased in GHR-/- mice.
Table 9. Proteins significantly different in GHR-/- compared to WT mice (p<0.01)

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein ID</th>
<th>Isoform #</th>
<th>Ratio (KO/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>apoA4</td>
<td>3</td>
<td>↑1.7</td>
</tr>
<tr>
<td>3</td>
<td>apoA4</td>
<td>4</td>
<td>↑1.9</td>
</tr>
<tr>
<td>12</td>
<td>RBP-4</td>
<td>1</td>
<td>↑1.8</td>
</tr>
<tr>
<td>13</td>
<td>RBP-4</td>
<td>2</td>
<td>↑3.0</td>
</tr>
<tr>
<td>14</td>
<td>RBP-4</td>
<td>3</td>
<td>↑4.2</td>
</tr>
<tr>
<td>Total</td>
<td>apoA4</td>
<td>Sum</td>
<td>↑1.6</td>
</tr>
<tr>
<td>4</td>
<td>apoE</td>
<td>1</td>
<td>↓0.12</td>
</tr>
<tr>
<td>6</td>
<td>apoE</td>
<td>3</td>
<td>↓0.40</td>
</tr>
<tr>
<td>7</td>
<td>apoE</td>
<td>4</td>
<td>↓0.60</td>
</tr>
<tr>
<td>8</td>
<td>apoA1</td>
<td>2</td>
<td>↓0.83</td>
</tr>
<tr>
<td>15</td>
<td>RBP-4</td>
<td>5</td>
<td>↓0.45</td>
</tr>
<tr>
<td>Total</td>
<td>apoE</td>
<td>Sum</td>
<td>↓0.61</td>
</tr>
</tbody>
</table>

Spot # corresponds to Figure 49; isoform # corresponds to Figure 15 in chapter III. The ratio of KO/WT was calculated as the mean value of KO/mean value of WT (all samples regardless of gender). ‘↑’ means up-regulation and ‘↓’ means down-regulation. KO: GHR-/- mice.

Figure 51. Cropped gel image from a representative mouse in each group showing increased intensity of apoA4 and decreased intensity of apoE in GHR-/- versus WT mice. Spots 1 and 2 increased as females aged but did not change during male aging. ApoA4 spots are in the oval circle and apoE spots in the squares. Protein # corresponds to Figure 49.
Figure 52. 3-D view of intensity of apoA4 (spots 1-3) and apoE (spots 4, 6 and 7). Spot intensity was converted into topographic view of a peak by PDQuest. Protein # corresponds to Figure 49. ApoA4 increased, whereas apoE decreased in GHR-/- versus WT in both genders. For apoA4, spots 1 and 2 showed no change during aging in males, but increased during aging in females. For apoE, spot 4 increased in females, and spot 7 was the lowest in female GHR-/- compared the other 3 groups.
Figure 53. 3-D view of intensity of apoA1 (spots 8-11). Protein # corresponds to Figure 49. Cropped images of original gel are shown in the center at the bottom of each graph. Spot 8 decreased in GHR−/− mice. Spots 8-11 showed increase during aging in females, but unchanged in males.
Figure 54. 3-D view of intensity of RBP-4 (spot 16), TTR (spot 17) and Alb (spots 19 and 20). Protein # corresponds to Figure 49. Spot 16 increased in female compared to male mice. Spot 17 increased in female aging, spots 19 and 20 decreased in female aging with no change or fluctuation in male aging.
Figure 55. 3-D view of intensity of RBP-4 (spot 14). The number of the spot corresponds to that in Figure 49. This spot decreased in female aging but fluctuated in male aging.
Figure 56. 3-D view of intensity of RBP-4 (spots 15) and Alb (spot 18). Protein # corresponds to Figure 49.
Figure 57. 3-D view of intensity of RBP-4 (spot 12) and Alb (spot 21). Protein #
corresponds to Figure 49.
Figure 58. 3-D view of intensity of RBP-4 (spot 13). Protein # corresponds to Figure 49. This spot increased in GHR-/- compared to WT mice.

**Plasma proteins that exhibited significant interaction between gender and genotype**

Four proteins showed a significant interaction between gender and genotype (p<0.01), i.e., whether levels in GHR-/- were higher or lower than WT depended on gender (Figure 59). Spot 7, an apoE isoform, had the same level between GHR-/- and WT in males, but was lower in GHR-/- females relative to controls (Figure 59 and
Figure 52). Spot 12, an RBP-4 isoform, showed no difference between GHR-/- and WT in males, but was higher in GHR-/- females versus controls (Figure 59 and Figure 57). Two Hp isoforms, spots 23 and 24, were lower in male GHR-/- but were higher in GHR-/- females versus controls (Figure 59 and Figure 60).

Figure 59. Plasma proteins that exhibited significant interaction between gender and genotype (p<0.01). Protein # corresponds to Figure 49.

Figure 60. 3-D view of intensity of Hp (spots 23 and 24). Protein # corresponds to Figure 49. These images are from mice at 24 months of age.
Discussion

*Body composition, glucose and insulin*

All animals gained weight from 9 to 16 months, but males had a slight weight loss at 24 months. This was consistent with the data presented regarding WT male mice during aging in chapter III. Interestingly, in females, body weight plateaued at 24 months, suggesting a gender-specific effect of aging on body weight. Since females in general live longer than males (Waldron & Johnston, 1976), the protection from weight loss at an old age may imply that females are more ‘fit’ than males at advanced ages.

GHR-/- mice had about 50% reduction in body weight compared to WT mice. There was no gender difference in GHR-/- mice, but female WT mice weighed less than WT males at 9 and 12 months, although the difference was diminished at 16 and 24 months. This suggested that an intact GH signaling system had a sexually dimorphic effect on body weight. For example, GH secretion is pulsatile in males but less so in females (Leppaluoto et al., 1975; Saunders et al., 1976; van den Berg *et al*., 1996), and estrogen inhibits GH action in females (Meinhardt & Ho, 2007), contributing to reduced lean mass in females. Interestingly, female WT mice weights caught up with WT males at old ages, perhaps due to reduced GH and reduced estrogen (in females) at older ages.

GHR-/- mice had decreased lean mass %, increased fat mass %, and no change in fluid mass % compared to WT controls, as has been reported previously (Berryman *et al*., 2004). Again, there was no gender difference found in GHR-/- mice. In contrast, female WT mice had a reduced lean mass relative to WT males, suggesting that an intact GH signaling system is required to maintain a gender-dependent body composition.
Fasting glucose levels were lower in GHR-/- than WT mice at an adult age (9 months), but were similar at an old age (24 months). Fasting insulin levels were lower in GHR-/- mice at both ages. These data were consistent with previous studies in which GHR-/- mice were slightly hypoglycemic but normalized with age (Coschigano et al., 2003). GHR-/- mice also had increased insulin sensitivity throughout their lifespan (Al-Regaiey et al., 2005; Coschigano et al., 2003). Interestingly, females had lower insulin than males, similar to a previous report (Guo et al., 2005).

In summary, body weight and composition not only revealed a well-known genotype difference (more fat and less lean in GHR-/- compared to WT mice), but also a gender difference found only in WT (females had less lean mass) and not GHR-/- mice. Blood glucose and insulin, on the other hand, showed a similar gender difference in both genotypes (lower insulin in females), in addition to the well-known genotype difference (lower insulin and glucose in GHR-/- mice).

**Plasma protein characteristics**

**ApoA4 increased in GHR-/- versus WT mice and increased during aging in females but not in males**

ApoA4 is in theory 43 kDa with a pI of 5.27. The five identified isoforms in this study were ~43 kDa with pI ranging from 5.64-5.9 (Figure 15 in chapter III, also see appendix A). ApoA4 is known to be phosphorylated, but this is not likely the only reason for the observed pI difference, as phosphorylation shifts spots to more acidic rather than basic pI.
Three out of four isoforms showed interesting changes. Two of them increased in GHR-/- mice (spots 2 and 3), as did total apoA4. In addition, spots 1 and 2 increased during aging in female compared to male mice of both genotypes.

ApoA4 is secreted by liver and intestine and is a major component of high-density lipoprotein (HDL). Most plasma apoA4 is associated with lipids (84%), about 12% associated with apoA1 and only 4% in free form (Ezeh et al., 2003). It is involved in chylomicrons and very low-density lipoprotein (VLDL) secretion and catabolism. In addition to cholesterol transportation and metabolism, apoA4 is also an anti-oxidant protein that removes superoxides and lipid hydroperoxide (Ostos et al., 2001; W.-M. R. Wong et al., 2007). It has anti-atherosclerosis effect and reduces inflammation (Recalde et al., 2004). Over expression of apoA4 in apoE-/- mice protect these mice from diet-induced atherosclerosis (Duverger et al., 1996). ApoA4 is a marker for coronary heart disease, with lower levels indicating higher risk (Warner et al., 2001). Patients with coronary artery disease have lower levels of apoA4 (Kronenberg et al., 2000).

It is possible that increased apoA4 levels in GHR-/- mice conferred protective effects against oxidative stress and heart diseases on these long-lived mice. It is also known that females generally live longer than males, and that males have higher rates of mortality from cardiovascular diseases (Waldron & Johnston, 1976). Therefore, the increase during aging of apoA4 in females, but not in male mice, may further imply a beneficial effect of circulating apoA4 in females.
ApoE decreased in GHR-/- versus WT mice; one isoform increased in female versus male mice; and one isoform showed interaction between genotype and gender

Of the five apoE isoforms identified (Figure 15 in chapter III), three of them showed changes in this study. ApoE has a theoretical Mw of ~34 kDa and the observed Mw on the gel was ~34 kDa. An additional apoE spot was also detected (spot 4 in Figure 49) which was ~38 kDa. The PTM for the size difference of this isoform is not clear. Although not reported in mice, in human plasma, apoE is glycosylated and glycated, which could in theory result in protein spots of slightly different Mws and pIs on a 2D gel.

GHR-/- mice had decreased apoE isoforms (spots 6 and 7) as well as total apoE. ApoE is associated with HDL and VLDL. Reduced apoE in GHR-/- mice may therefore reflect the fact that they have reduced plasma cholesterol levels (both HDL and LDL) compared to WT mice (A. Bartke et al., 2004; E. Egecioglu et al., 2005; Masternak et al., 2005a). This is consistent with the previous result where bGH mice had increased apoE in all isoforms (chapter IV). Hypophysectomized (Hx) rats had decreased apoE which normalized upon GH replacement (Frick et al., 2002; Jan Oscarsson et al., 1991b; Sjoberg et al., 1994a; Sjoberg et al., 1994b). In this regard, apoE shows a positive response to GH.

One isoform (spot 4, the 38kDa isoform) of apoE was increased in females, although total apoE levels did not differ. It is possible that this specific isoform, or the PTM responsible for the Mw increase, is regulated by gender differences. Female rats have been found to have higher levels of apoE than males (Patsch et al., 1980). In
humans, apoE is involved in age-related diseases including Alzheimer’s disease, dementia, cognitive decline, stroke and cardiovascular diseases in a gender-specific manner (Frikke-Schmidt et al., 2004; Galasko et al., 2007; Ghebremedhin et al., 2001; Gromadzka et al., 2007; Mortensen & Hogh, 2001; Rosvall et al., 2008). For example, apoE allele ε4 in men is associated with increased mortality whereas ε2 in women is associated with decreased mortality (Rosvall et al., 2008). ApoE ε4 is also an independent predictor of death within 1 year after stroke incidence in men but not in women (Gromadzka et al., 2007). However, ε4 allele is associated with age-related cognitive decline in women but not in men (Mortensen & Hogh, 2001). An association between the ε4 allele and Alzheimer’s disease-related senile plaques was found in men but not women over 80 years of age (Ghebremedhin et al., 2001). Thus, apoE affects males and females differently.

Different alleles of apoE could result in different isoforms on a 2-D gel. However, it is not known if the C57Bl/6J mice used in our study have different alleles of apoE. Theoretically, these in-bred mice should be genetically identical; therefore they should have the same allele of apoE. Thus the isoforms observed on 2D gels are likely due to PTM instead of different alleles.

Interestingly, one isoform (spot 7) of apoE showed significant interaction between gender and genotype such that there was no gender difference in WT, but was reduced in GHR-/- females, suggesting an intact GH signaling is required for the gender difference of this apoE isoform.
ApoA1 decreased in female mice, increased during aging in females but not in males

Six isoforms of apoA1 were identified (Figure 15 in chapter III). Theoretical Mw and pI of apoA1 are ~28 kDa and ~5.4, respectively. Observed values of the five isoforms on our gels were 28 kDa and pI ranging from 5.73-6.4.

Spot 10, an apoA1 isoform, decreased, although total apoA1 did not change in GHR-/- mice versus controls. Total apoA1 decreased in female mice versus male mice of both genotypes. Four isoforms (spots 8-11) increased during aging in females but did not change in males. ApoA1 associates with HDL, and is therefore considered a beneficial marker for cardiovascular disease risk. Even though total apoA1 decreased in females, it showed an increased trend during aging, possibly indicating an increased protective effect.

GHR-/- mice have reduced HDL (E. Egecioglu et al., 2005; Liu et al., 2004). However, GHR-/- mice have normal total apoA1 in this study, although one isoform decreased compared to WT mice. Laron Syndrome patients have normal levels of HDL (Shechter et al., 2007). Acromegalic patients have reduced HDL (Delaroudis et al., 2008). In agreement, bGH mice had decreased apoA1 relative to WT controls (chapter IV). It seems that excess GH leads to unfavorable HDL and apoA1 levels, whereas lack of GH action results in normal apoA1 and normal or reduced HDL levels.

RBP-4 showed an isoform-specific change in gender, genotype differences, as well as an interaction between gender and genotype

Seven isoforms of RBP-4 were observed (Figure 15 in chapter III) and discussed in chapter IV. Three isoforms of RBP-4, spots 12, 13 and 14, increased while one isoform,
spot 15, decreased in GHR-/- mice. Interestingly, spots 12, 13 and 14 were the larger isoforms (22kDa) whereas spots 15 and 16 were the shorter isoforms (19kDa). Thus, 22kDa forms increased in GHR-/- and the 19kDa forms decreased in GHR-/- . This is consistent with previous chapter IV in which bGH mice had increased 19kDa and decreased 22kDa RBP-4 isoforms. Thus, the PTM event that regulates this process may be subjected to GH regulation. Regarding gender differences, spot 16 (19kDa) increased while spot 14 (22kDa) decreased in females. Spot 14 also showed a decrease by age in females, but not in males. On close examination, spots 12-14, the 22kDa isoforms, increased in GHR-/- versus WT mice and was more pronounced in females than males (Figure 61). Similarly, for spots 15 and 16, the 19kDa isoforms, decreased in GHR-/- versus WT and was greater in females than males. Since males have a pulsatile GH secretion pattern, whereas females have a more constant pattern, this suggests that the female GH pattern is more potent in increasing the 19kDa and decreasing the 22kDa RBP-4 (or the male pattern reverses it). When GH is absent such as in GHR-/- mice, the difference between male and female is diminished in these RBP-4 isoforms (Figure 61).

Based on these data, it is possible that the relative proportion of 22 and 19kDa RBP-4 isoforms is regulated by GH in terms of both the amount and secretion pattern in circulation. In this regard, bGH mice have constitutively elevated GH levels, therefore, pronounced effect on this regulation (increasing the 19kDa and decreasing the 22kDa isoforms, see chapter IV for data). Thus, it is important to differentiate between RBP-4 isoforms as a predictor of GH effects and the effects on aging.
RBP-4 is secreted by liver and fat, and more so in visceral than subcutaneous adipose depot (Kloting et al., 2007). It is associated with non-alcoholic fatty liver (Seo et al., 2008), insulin resistance (Reinehr et al., 2008) and type 2 diabetes (Chang et al., 2008; K. Takebayashi et al., 2007b). Total RBP-4 levels tend to be lower in women than men (Ichihara et al., 2004). Despite the obesity seen in GHR-/- mice, they did not have increased total RBP-4 compared to WT mice. This may be because of the contribution of liver-derived RBP-4, or that the excess fat of GHR-/- mice is subcutaneous rather than visceral.
One isoform of TTR showed interaction between gender and aging

Nine TTR spots were identified (Figure 15 in chapter III). TTR is in theory 13.6 kDa (without the signal peptide) with a pI of 5.77. Possible PTMs of TTR are known to exist in human plasma including phosphorylation, cysteinylation, dihydroxylation and glutathionylation (Hutchinson et al., 1996; Terazaki et al., 1998; Zheng Wang et al., 2004). The details of each isoform were discussed in chapter III. This paper will focus only on the isoform that changed in this study (spot 17, ~21 kDa).

This TTR isoform (spot #17) showed an increase with age in females more so than males. Interestingly, this was also the TTR isoform that increased during aging in male WT mice (See chapter III). This seems to be paradoxical to the notion that females live longer. One possibility is that this TTR is actually protective and induced at older ages to counteract some of the damage imposed by aging.

Hp decreased in females versus males and showed an interaction between genotype and gender

Of the four Hp isoforms identified (Figure 15 in chapter III; also see discussions in chapters III and IV), one (spot 22) decreased in females, two (spots 23 and 24) showed interaction between genotype and gender such that they decreased markedly in females in WT, but did not change as much in GHR-/- mice.

Hp is an acute phase protein indicating inflammation. Hp binds to heme to reduce oxidative stress. Thus, reduced levels of Hp may indicate reduced need of Hp to bind heme. Incidentally, females did show reduced hemoglobin beta levels in plasma (see below). Therefore it is possible that female mice had reduced levels of oxidative stress
related to heme release. Also, in WT mice, this gender difference was obvious, however, in GHR-/- mice, male and female mice both had low levels of Hp (compared to WT male) and did not differ significantly. It was shown in chapter IV that bGH mice had increased Hp relative to WT controls. Together, these data implied that low levels of Hp indicated reduced inflammation in GHR-/- (both male and female) versus WT controls; and in WT mice, lower Hp levels indicate reduced inflammation in females. This could be one of the reasons that GHR-/- mice are long-lived, bGH mice short-lived and that females are usually longer-lived than males.

**Hbb decreased in females**

There were 4 spots identified as Hbb (Figure 15 in chapter III; also see appendix A for MS/MS matching). Hbb is in theory 15.7 kDa with a pI of 7.26. The identified four isoforms were ~12 kDa with pI ranging from 7.24 to 7.96. Therefore isoform 1 (Figure 15) is likely the non-modified Hbb. Hbb is known to be phosphorylated, although this is not likely the PTM for other three isoforms which have more basic pls. Also, the PTM responsible for the reduced Mw is not clear. Two of them, spots 26 and 27 (isoforms 1 and 3 in Figure 15), decreased in females (Table 8).

Hbb is a subunit of hemoglobin, the major protein in red blood cells. The presence of Hbb in the plasma may be due to blood cell lysis. It is not clear why female mice have lower Hbb. However, this may indicate that females have less oxidative stress due to heme release.
Alb decreased in females versus males and showed an interaction between gender and aging

As discussed in chapter III, the Alb isoforms seen on our 2D gels are most likely Alb fragments, and the PTMs for these fragments resulting in various Mw and pI are not clear. One isoform (spot 25, 12kDa) decreased in females. Two isoforms (spots 28 and 29; 9kDa) increased by age in females but not males, whereas four isoforms (spots 18-21; 16kDa) decreased by age in females but not males. It is difficult to explain the complex behavior of these albumin fragments. One possibility is that one or more theoretical proteases cleave albumin in plasma and act differentially between male and female mice. It is possible that this activity changes during aging between genders as well. Further studies are needed to confirm the albumin fragmentation pattern in mice during aging and its physiological significance.
Conclusion

This study has found that GHR-/- mice have lower glucose (in adult but not old mice), lower insulin, reduced body weights and lean mass, but increased fat mass. WT mice showed a gender difference in lean mass (reduced in females); however, this gender difference was diminished in GHR-/- mice, suggesting that GH is important in maintaining gender-specific body composition.

Biomarkers of lack of GH action as well as gender are summarized in Table 10.

Table 10. Biomarkers indicative of lack of GH action and gender difference

<table>
<thead>
<tr>
<th>Biomarkers indicating lack of GH</th>
<th>Gender differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in GHR-/- apoA4 (spots 2 and 3; total)</td>
<td>Decrease in GHR-/- apoE (spots 6 and 7; total)</td>
</tr>
<tr>
<td>RBP-4 (spots 12-14, 21kDa isoforms)</td>
<td>RBP-4 (spot 15, 19kDa isoform)</td>
</tr>
<tr>
<td></td>
<td>RBP-4 (19kDa isoform spot 16 and total)</td>
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<tr>
<td></td>
<td>RBP-4 (spot 14, 21kDa isoform)</td>
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<tr>
<td></td>
<td>Alb (spot 22)</td>
</tr>
<tr>
<td></td>
<td>apoA1 (total)</td>
</tr>
<tr>
<td></td>
<td>Hbb (spots 26 and 27)</td>
</tr>
<tr>
<td></td>
<td>Hp (spot 25)</td>
</tr>
</tbody>
</table>

Spot # corresponds to Figure 65.

One important finding is that GHR-/- mice have elevated apoA4 levels compared to WT mice, indicating an enhanced cardiovascular protective effect of apoA4 in this long-lived mouse. Interestingly, although female mice (both genotypes) do not have higher levels of apoA4, they do show an increased apoA4 toward old age versus no change during aging in male mice; further, females also have increased apoA1 during aging in contrast to no change in male aging. This is possibly one of the reasons females have reduced cardiovascular mortality and live longer than males.
In contrast to bGH mice, which have increased cholesterol and apoE, GHR-/ mice have decreased cholesterol and apoE. Thus apoE appears to be a sensitive marker for GH action involving lipid transportation and metabolism.

Expectedly, gender differences exist in plasma proteins. Total RBP-4 increased in females by 20%, and total apoA1 decreased by 15%. However, although a 19kDa isoforms of RBP-4 increased, another 22kDa isoform decreased in female versus male mice, similar to isoform changes in bGH compared to WT mice. This suggests a role of GH secretion pattern on PTM regulation of RBP-4. Total levels of apoE, clusterin and Hp do not change between genders, but specific isoforms show up or down-regulation in female versus male mice (Table 10). Further, females show a different aging profile on certain proteins from males. For example, apoA1 and apoA4 increase during female aging but not male aging, possibly indicating better cardiovascular protection as mice age in females. Two albumin fragments increase, whereas five albumin fragments decrease during female aging compared to little age-related changes in males. All these gender differences are independent of genotype, i.e., GHR-/ and WT mice show similar gender-specific differences.

Interestingly, some of the gender differences observed were genotype-dependent (Hp spots 23 and 24, apoE spot 7 and RBP-4 spot 12), implying an interaction of GH with gender. GH itself is secreted in a sexually dimorphic manner (MacLeod et al., 1991). GH also affects liver sexually dimorphically (Norstedt & Palmiter, 1984; D. J. Waxman, 2000; David J. Waxman & O'Connor, 2006; Wiwi & Waxman, 2004). Many of the plasma proteins are secreted by liver, and liver is known to express genes in a
sexually dimorphic manner. For example, drug metabolizing genes, including cytochromes P450 (CYPs), sulfotransferases, glutathione-S-transferases and UDP-glucuronosyltransferases (David J. Waxman & Holloway, 2009). More than 1000 genes have been identified in mouse and rat livers with sexually dimorphic expressions, substantially affecting liver metabolic function and pathophysiology (David J. Waxman & Holloway, 2009). The sexual dimorphism of liver gene expression is regulated by the GH secretion pattern, with pulsatile in males vs. more continuous in females. Liver STAT5b is phosphorylated by GH in male but not female rats (D. J. Waxman, 2000). Pulsatile GH causes phosphorylation of STAT5b but continuous GH actually downregulates STAT5b signaling in rat liver (D. J. Waxman, 2000). Hepatic nuclear factor (HNF)-4α also responds to GH in a sexually dimorphic manner, thereby possibly mediating the sex-dependent effects of GH on the liver (Wiwi & Waxman, 2004). These studies demonstrate that sexually dimorphic pattern of GH is responsible for the sexual dimorphism seen in liver gene expressions. It will be important to establish if the plasma proteins (i.e., Hbb) observed in this study are regulated by GH, more specifically, through STAT5b and/or HNF-4α. Also, in the case of isoform-specific changes, how PTMs of the plasma proteins (i.e., RBP-4) are regulated by GH signaling. Thus future studies are needed to reveal the functional inference of these proteins or specific isoforms related to GH and gender.
CHAPTER VI. BIOMARKERS OF GH AND IGF-1 DOPING: PROTEOMIC ANALYSIS OF PLASMA FROM WILD TYPE MICE INJECTED WITH GH AND IGF-1

Abstract

Recombinant human growth hormone (rhGH) has been and continues to be abused by athletes and is an important issue on the agenda of World Anti-Doping Agency (WADA). Due to the short half-life, pulsatile secretion pattern, and sequence identity to endogenous hGH, rhGH is very difficult to detect. Efforts have been made to discover and validate biomarkers of hGH that have relative long half-lives. A combination, rather than a single biomarker, may provide an accurate and sensitive assay to detect hGH doping.

Moreover, insulin-like growth factor-1 (IGF-1) is also abused by athletes and there is currently no method to detect IGF-1 doping. In order to complement the studies ongoing in the rhGH doping area, we set out to establish GH and IGF-1 doping biomarkers in mice. Thus, mice were injected subcutaneously with recombinant bovine (b) GH or rhIGF-1 for two weeks followed by one additional week of a drug-free washout period. It is important to be able to detect GH or IGF-1 doping after a period of drug-free washout because athletes normally will stop using drugs when the competition is up-coming. Plasma proteins were collected after the drug treatment or washout period and resolved by two-dimensional electrophoresis (2-DE). Proteins that showed significant difference with GH or IGF-1 treatment were identified by mass spectrometry (MS) and MS/MS. We have found several markers of GH action in the plasma of GH treated mice:
namely three isoforms of haptoglobin (Hp), one isoform of retinol-binding protein-4 (RBP-4), six isoforms of albumin, one isoform of α-2 macroglobulin (a2m) and one isoform of serum amyloid A-1 (SAA-1), all of which increased in the group receiving GH treatment with a one week washout period. Also, one isoform of apolipoprotein A-4 (apoA4) was found to be significantly decreased following GH treatment and in the one week washout period. Additionally, ApoE and clusterin showed significant changes after GH treatment but returned to control levels after 7 days of washout; thus, their response to GH was not long lasting and did not warrant further study. In terms of biomarkers for IGF-1, two isoforms of Hp and TTR, as well as six isoforms of albumin, increased after IGF-1 treatment and in the washout period. Important to the study of attempting to separate GH versus IGF-1 action, we found several plasma proteins to be differentially regulated by GH and IGF-1, including apoE, which was increased by GH but not affected by IGF-1, and one isoform of TTR and four isoforms of albumin which were increased by IGF-1 but not affected by GH. If these novel biomarkers for GH and IGF-1 are verified in humans, then perhaps novel human markers of GH vs IGF-1 action will be established and commercial kits to detect GH or IGF-1 abuse will be generated.
Introduction

Growth hormone (GH) is secreted by somatotrophs of the anterior pituitary gland and has a central role in stimulating longitudinal growth and regulating metabolism of protein, carbohydrates, lipids and mineral (Baumann; Kopchick & Andry, 2000). GH promotes bone, cartilage and muscle growth as well as fat degradation; it also induces insulin resistance and hyperglycemia (LeRoith & Yakar, 2007). Recombinant human GH (rhGH) is abused by athletes to increase muscle, degrade fat and to enhance performance. However, the positive role of short-term GH administration in muscle mass, strength and endurance as well as athletic performance have been brought into question as positive performance outcomes of GH administration have not been reported (Berggren et al., 2005; Ehrnborg et al., 2005). Still, rhGH abuse is rampant among athletes and is on the prohibited substance list by World Anti-Doping Agency (WADA) (WADA, 2009).

Injected rhGH is difficult to detect in blood for three reasons. First, rhGH is indistinguishable from endogenous GH. Second, GH has a relatively short half-life of ~15 minutes (R. W. Holl et al., 1993). Exogenous GH is cleared quickly from circulation and injected rhGH is also quickly cleared from the blood (Hashimoto et al., 2000; Owens et al., 1973; Jennifer D. Wallace et al., 2001). Third, GH secretion from pituitary somatotrophs is pulsatile, especially in males (Goldenberg & Barkan, 2007), thus making it hard to determine the exact time for GH measurement. Therefore, it is difficult to directly detect GH levels in the blood.

GH induces the production of insulin-like growth factor-1 (IGF-1) in the liver as well as many GH responsive organs. IGF-1 mediates many of the actions of GH, e.g.,
muscle hypertrophy, bone growth and fluid retention. IGF-1 has been used traditionally as a marker for GH, being positively correlated with the level of GH. However, in some cases, plasma IGF-1 levels cannot properly reflect blood GH levels. For example, some acromegalic patients have high levels of IGF-1, but GH levels are normal. Thus, the correlation of GH and IGF-1 is poor. Also, after treatment to normalize GH, IGF-1 does not always normalize (Barkan, 2007; Clemmons, 2006; Popovic, 2005; Stonesifer et al., 1981). Thus, other plasma biomarkers for GH action would be valuable.

In addition, because of the anabolic effect of IGF-1, it is being abused in the athletic world as well (Guha et al., 2009; R I G Holt, 2008). RhIGF-1 is FDA-approved for the treatment of children with short stature caused by primary IGF deficiency. Currently, there is no technology to detect IGF-1 abuse. Therefore, it is important to find biomarkers for IGF-1.

Because GH induces IGF-1, it is difficult to dissect out the specific effects of GH or IGF-1. This has been an enigma in the GH/IGF-1 field, i.e. separating the molecular action of GH and IGF-1.

The mouse is a good model to study human biology because it shares high homology with humans at both the genomic and proteomic levels. In this study, three-month-old normal male mice were injected daily with recombinant bovine growth hormone (bGH) or recombinant human (h) IGF-1 for 14 days. Two additional groups of mice were given a 7-day washout period following these injections. Then, the immediate effect as well as a washout effect was observed by analyzing the mouse plasma proteome via two-dimensional electrophoresis (2-DE). Plasma protein levels that changed
significantly by GH or IGF-1 in their washout period were identified by mass spectrometry (MS) and MS/MS. Several plasma biomarkers were identified. Hopefully, they will have the potential to indicate GH and IGF-1 doping in humans as well.
Materials and Methods

*Experimental animals and treatment protocol*

Three-month old male C57 BL/6J mice were used for these studies. These represent relatively young adult animals. Mice were housed 2-3 per cage at room temperature (22 °C) in a 12-hour light, 12-hour dark cycle. Mice were fed *ad lib* with a standard chow diet.

Bovine (b) GH and rhIGF-1 were administered to the mice via subcutaneous injection daily for a total of 21 days. Purified recombinant bGH was a gift from Monsanto (St Louis, MO) and was dissolved in standard phosphate buffered saline (PBS). RhIGF-1 was a gift from Tercica Inc. (Brisbane, CA) and dissolved in a solution called ‘vehicle’ containing 9 mg/mL benzyl alcohol, 5.84 mg/mL sodium chloride, 2 mg/mL polysorbate 20, and 0.05M acetate at a pH of approximately 5.4. Mice were divided randomly into 5 groups (n=7 for each group). bGH was injected once per day at a dose of 5ug/gram of body weight as previously described (List et al., 2009). IGF-1 was administered twice per day at a dose of 5ug/gram body weight for a total of 10ug/gram body weight per day to prevent hypoglycemic shock for the animals. To control for the stress levels as well as the different solutions used for bGH and rhIGF-1 preparation, each mouse was injected three times daily (one for PBS or bGH, two for vehicle or IGF-1) as summarized in Table 11 and Figure 62. All animals were sacrificed at the end of the study. Animal protocols were approved by Ohio University’s Institutional Animal Care and Use Committee.
Table 11. GH and IGF-1 treatment scheme

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>PBS+vehicle(x2)</td>
<td>PBS+vehicle(x2)</td>
<td>PBS+vehicle(x2)</td>
</tr>
<tr>
<td>2. GH</td>
<td>PBS+vehicle(x2)</td>
<td>GH+vehicle(x2)</td>
<td>GH+vehicle(x2)</td>
</tr>
<tr>
<td>3. GH washout</td>
<td>GH+vehicle(x2)</td>
<td>GH+vehicle(x2)</td>
<td>PBS+vehicle(x2)</td>
</tr>
<tr>
<td>4. IGF-1</td>
<td>PBS+vehicle(x2)</td>
<td>PBS+IGF-1(x2)</td>
<td>PBS+IGF-1(x2)</td>
</tr>
<tr>
<td>5. IGF-1 washout</td>
<td>PBS+IGF-1(x2)</td>
<td>PBS+IGF-1(x2)</td>
<td>PBS+vehicle(x2)</td>
</tr>
</tbody>
</table>

Figure 62. GH and IGF-1 treatment scheme.

Body composition measurement

Body composition was measured using mq 7.5 NMR analyzer the Bruker Minispec (The Woodlands, Tx) as described previously (List et al., 2009; Palmer et al., 2009) before and after the 21-day injection. Each animal was measured twice and the mean value was determined. The data are given as change in grams (g) for lean mass, fat mass and fluid mass.
**Fasting blood glucose**

Mice were fasted for 12 hours overnight and blood was collected with heparinized capillary tubes by tail tip clipping. Whole blood was centrifuged at 7000 x g for 10 minutes at 4 °C and the resulting plasma was stored at -80 °C. Fasting glucose was measured with the first drop of blood from tail tip at 9AM after 12h over night fasting using ONE TOUCH glucometer from Lifescan (Milpitis, CA).

**2-DE and quantification of proteins**

See chapter III.

**Statistical analysis**

All statistical analyses were performed using SPSS 14.0 software (Chicago, IL). Data for body composition and fasting glucose were subjected to one-way ANOVA test using LSD test for Post Hoc comparison (p<0.05 considered significant). Log-transformed data for proteomics were subjected to one-way ANOVA with p<0.01 considered significant, then Post Hoc comparison using LSD method was applied to detect between group difference (p<0.05). All data were presented as mean ± SEM.

**Protein identification by mass spectrometry (MS), MS/MS, and liquid chromatography (LC)-MS/MS**

See chapter III.
Results

Body composition

After 3 weeks of treatment, the 5 groups of mice had significantly different weight changes (p=0.009). All GH/IGF-1 treated mice had increased weight gains compared to controls (Figure 63). The GH treated group gained the most weight, followed by IGF-1 group. Interestingly, whereas the IGF-1 washout group had slightly decreased weight gains relative to the IGF-1 group, the GH washout group had marked decreased weights compared to the GH group. However, the weight gains were still higher than controls, which suffered from weight loss, presumably due to stress from daily injections.

Figure 63. Body weight and body composition change after the injections. Different letters denote significant differences (p<0.05). GHw: GH washout; IGFw: IGF-1 washout.
Lean mass gain was significantly different among the 5 groups (p=0.007). Other than controls and the GH washout group, all treated mice had a positive lean mass gain (Figure 63). The GH group had the largest lean mass gain, although the effect was diminished after 1 week of washout; whereas both IGF-1 and IGF-1 washout groups showed positive lean mass gain. Fat gain was not significantly different among 5 groups (p=0.13), although a trend in increased fat loss was observed in GH group (Figure 63). Fluid gain was significantly different among 5 groups (p=0.016). Both GH and IGF-1 increased fluid gain compared with controls (Figure 63), whereas this fluid retention effect was diminished in the washout groups.

Taken together, the weight loss of control mice was attributed to lean and fluid mass loss, not fat loss. The weight gain of GH and IGF-1 groups was due to lean mass and fluid gain; and the weight gain of IGF-1 washout group was primarily due to lean mass gain. In summary, GH treatment increased lean and fluid mass, decreased fat mass; however, the effect was temporary. In contrast, IGF-1 increased lean and fluid mass, did not decrease fat mass, and maintained lean mass after a 7-day washout period.

**Fasting blood glucose**

Fasting blood glucose was not significantly different among the 5 groups (p= 0.195, Figure 64).
Figure 64. 12-hour fasting glucose levels. No significant difference was found among five groups.

**Plasma proteins altered by GH/IGF-1**

A total of 165 protein spots were detected by 2-DE. Among them, a subset of spots showed significantly different intensities among the 5 groups of mice. Some had markedly altered levels by GH and/or in the GH washout period, which could serve as potential biomarkers for GH doping. Some of them were altered significantly by IGF-1 and/or the IGF-1 washout period, and therefore are potential biomarkers for IGF-1 doping. Very importantly, some proteins were differentially altered by GH and IGF-1, illustrating specific effects of GH and IGF-1 on plasma proteins. *Figure 65* is an annotated 2D gel image with the selected spots of interest. Notice that multiple spots were identified as the same protein probably due to post-translational modifications (PTMs) of the identified protein. The nature of these PTMs has not been identified.
Figure 65. 2-D gel map of proteins that changed significantly during GH or IGF-1 treatment or after the respective washout period. Mw: molecular weight; pI: isoelectric focusing point; apoA4: apolipoprotein A-4; a2m: alpha-2 macroglobulin (35kDa subunit); Clu: clusterin; apoE: apolipoprotein E; RBP-4: retinol-binding protein-4; TTR: transthyretin; Hp: haptoglobin (alpha-chain); SAA-1: serum amyloid protein A-1.

**Plasma proteins altered by GH**

The plasma levels of several proteins were altered by GH treatment and returned to control levels after the washout period. These included apoE, which increased, and clusterin, which decreased (Figure 66).
Figure 66. Proteins that responded to GH and returned to control levels after the GH washout period. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). A, apoE isoform (2) and total apoE (defined as sum of all four isoforms) increased in GH group but remained unchanged in GH washout group. B, four isoforms as well as total clusterin decreased in GH but remained unchanged in GH washout group. Protein names and isoform # corresponds to Figure 65. GHw: GH washout.

ApoE, in particular isoform 2, increased after GH injection; however, the levels were the same as controls after the 1 week of washout period (Figure 67). ApoE was not altered by IGF-1 injections.
Clusterin (isoforms 3-6) decreased immediately after GH treatment, but rose to control levels after the washout period (Figure 68).

Several plasma protein levels were changed following GH treatment but were found altered only after the washout period rather than immediately after GH treatment. These
included one isoform of TTR and apoA4 that decreased (Figure 69); Hp (Figure 71), RBP-4 (Figure 71), a2m (Figure 67) and SAA-1 (Figure 72) that increased (Figure 70), as well as multiple isoforms of albumin that increased (Figure 73).

TTR isoform 2 decreased after 1 week of the GH washout period. ApoA4 isoform 5 decreased after 1 week of GH washout. However, other isoforms of TTR or apoA4 were not changed.

Figure 69. Proteins that decreased after the GH washout period. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). Protein names and isoform # corresponds to Figure 65.

Figure 70. Proteins that were increased significantly after washout of GH injection (one-way ANOVA, p<0.01). Different letters denote significant differences (p<0.05). Protein name and isoform # corresponds to Figure 65. GHw: GH washout.

All three isoforms of Hp increased after the GH washout period (Figure 71). One isoform of RBP-4 also increased after GH washout (Figure 71). IGF-1 treatment increased Hp, but did not change RBP-4 (Figure 71).
Figure 71. 3-D view of intensity of Hp isoforms 1-3 and RBP-4.

SAA-1 was slightly decreased after GH treatment, but increased after the GH washout period (Figure 72).

Figure 72. 3-D view of intensity of SAA-1. Note that SAA-1 increased in the GH washout group.

Alb isoforms including spots 1-6 all increased after the GH washout period (Figure 73, Figure 75 and Figure 76).
Figure 73. Multiple albumin fragments were increased after washout of GH injection. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). Isoform # corresponds to Figure 65. GHw: GH washout.

Figure 74. 3-D view of intensity of Alb (spots 1 and 2) and TTR (spot 1). Isoform # corresponds to Figure 65.
Figure 75. 3-D view of intensity of Alb spots 3 and 4. Isoform # corresponds to Figure 65.
Figure 76. 3-D view of intensity of Alb (spots 5 and 6). Isoform # corresponds to Figure 65.

Plasma proteins altered by IGF-1

Several proteins increased by IGF-1 injection and remained at elevated levels after the washout period, including Hp, TTR (Figure 77), and multiple forms of Alb (Figure 79).
Figure 77. Proteins that were increased by IGF-1. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). Isoform # of Hp and TTR corresponds to Figure 65. IGFw: IGF-1 washout.

Hp isoforms 1 and 3 increased in the IGF-1 treated group and increased further after the washout period (Figure 71).

TTR isoforms 1 and 3 both increased after IGF-1 injection and isoform 1 increased further after the washout period (Figure 74). Isoform 3 remained elevated after the washout period (Figure 78).
Figure 78. 3-D view of intensity of TTR (spot 3). Isoform number corresponds to that in Figure 65.

Six Alb isoforms (1, 2, 4, 5, 7 and 9) all increased after IGF-1 injection and remained at the elevated levels after the washout period (Figure 74, Figure 75, Figure 76 and Figure 79).
Figure 79. Alb fragments that were increased by IGF-1. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). Isoform # corresponds to Figure 65. IGFw: IGF-1 washout.

Plasma proteins differentially altered by GH and IGF-1

Several proteins responded to GH and IGF-1 differentially, including apoE, TTR (Figure 80) as well as individual albumin fragments (Figure 81).

ApoE (isoforms 2 and 3 and total levels) increased by GH but not IGF-1 treatment (Figure 67). TTR (isoform 3) was not changed by GH but increased by IGF-1 treatment (Figure 78). Alb 1, 2 and 5 did not change after GH treatment but did increase after IGF-1 injections (Figure 74, Figure 75 and Figure 76).
Figure 80. Proteins that were changed by GH and IGF-1 differentially. Significant difference was found among three groups via one-way ANOVA (p<0.01). Different letters denote significant differences (p<0.05). Isoform # corresponds to Figure 65.

Figure 81. Alb fragments that were changed by GH and IGF-1 differentially. Significant difference was found among three groups via one-way ANOVA (p<0.01). Isoform # corresponds to Figure 65.
Discussion

Two weeks of GH or IGF-1 injections did not alter fasting glucose levels significantly. However, body weights and compositions changed. Mice gained lean mass with GH treatment but did not maintain this mass. In contrast, IGF-1 treatment was able to retain the lean mass gain even after the washout period. This is perhaps because the anabolic effect of GH is mediated through IGF-1, and IGF-1 has a much longer serum half-life than GH. Both GH and IGF-1 promoted body weight and lean mass gains, consistent with the well known anabolic effect of the GH/IGF-1 axis (Cheek & Hill, 1974; N. Moller & Jorgensen, 2009; Velloso, 2008).

Increased trend (not significant) in fluid gain in the GH and IGF-1 group indicates the fluid retention effect of GH (Carroll & Van den Berghe, 2001; Cleemans et al., 1987; O'Sullivan et al., 1994). GH and IGF-1 promote fluid retention by stimulating renin-angiotensin-aldosterone system which is central to fluid homeostasis (J. Moller et al., 1999).

Fat mass was slightly reduced, although not significantly, by GH. The fat decreasing effect was not seen after 7 days of washout. Apparently, the fat degrading effect was only induced by GH, not IGF-1, as IGF-1 group did not show a decrease in fat. This effect has been reported previously (Clark et al., 1996; Dubuis et al., 1996; Fielder et al., 1996).

Thus, the weight gain that we detected was due to increased lean and fluid mass and was not due to changes in fat. The GH group had a larger weight gain because of the
larger lean mass gain. However, during the GH washout period, a quick rebound of weight was seen when compared to mice in the GF-1 washout group.

In summary, the body composition data confirmed lean mass anabolic and fluid retention actions of GH/IGF-1, which normalized after a one-week washout period. An exception to this statement was the lean mass and body weight gain for IGF-1 washout group. These data were consistent with a previous study with GH injection in mice (List et al., 2009).

The levels of many plasma proteins were altered during GH or IGF-1 treatment. ApoE (isoform 2 as well as total levels) increased by GH injection, consistent with markedly increased apoE (all isoforms) that was found in bGH mice (chapter IV). ApoE has also been show to increase in individuals with acromegaly (Wildbrett et al., 1997). However, apoE levels returned to control levels after a one week washout period, indicating that apoE is an acute marker but not a long-lasting marker of GH action. ApoE is increased in rats with GH administration and this effect is not mediated by IGF-1 (Sjoberg et al., 1994b). GH has been shown to increase apoE by increasing the translation and secretion rate of apoE from hepatocytes (Sjoberg et al., 1994a).

ApoE is secreted primarily by the liver and functions to transport lipids via very-low-density lipoprotein (VLDL), LDL and high-density lipoproteins (HDL). The change of apoE by acute GH treatment therefore indicates an acute effect on lipoprotein metabolism. ApoE also is considered an antioxidant that helps to fight oxidative stress (Davignon, 2005; Miyata & Smith, 1996; Tarnus et al., 2009), reduces DNA damage
(Folkmann et al., 2007) and inflammation (Lynch et al., 2001). Thus, short-term GH treatment may improve resistance to oxidative stress by increasing apoE.

Similarly, clusterin (isoforms 3-6 as well as total levels) decreased by GH injection, also consistent with the lower levels found in bGH mice at young ages (chapter IV). Note that bGH mice expressed high levels of clusterin when they were older, which could indicate the accelerated aging phenotype of these mice (chapter IV). However, the physiology of this age-dependent increase in clusterin is complicated. Since bGH mice of 2 and 4 months, as well as GH-injected mice (at 3 months in this study) had lower levels, clusterin could be considered a negative marker of GH action. Unfortunately, the effect was short-lived as the GH washout group had normal levels of clusterin. Clusterin is also associated with HDL, therefore the reduced clusterin by GH may indicate a lipid profile change by GH.

One isoform of TTR (isoform 2) did not change immediately after GH injection, but decreased after a week of washout, suggesting TTR lowering effect of GH was not acute, but relatively long lasting. Consistently, bGH mice had lower levels of TTR than WT at all ages. Therefore, TTR, in particular, isoform 2, is a long-lasting (at least one week) biomarker for GH. As shown in chapter IV, chronic GH excess leads to reduced levels of eight TTR isoforms including the one that decreased in this study. Perhaps this isoform is the most sensitive to GH action.

One isoform of apoA4 (isoform 5) showed similar behavior, that is, decreasing after the GH washout period but not immediately after injections. This is in contrast with bGH mice, which did not show difference in any of the apoA4 isoforms compared to WT
mice. However, GHR-/- mice have increased apoA4 (2 isoforms as well as total). Therefore, apoA4 could be considered a negative marker of GH action.

Hp increased (isoforms 1-3 and total levels) in the GH washout group although not immediately after GH injections. bGH mice had increased Hp (all isoforms and total) compared to WT mice; therefore, Hp is a long-lasting biomarker of GH. As with bGH mice, this suggests that short-term treatment of GH also induces an inflammatory state in mice.

RBP-4 (one isoform of 19kDa) increased in the GH washout group although not in the GH group. This was consistent with data in chapter IV, in which bGH mice had increased RBP-4 19kDa isoforms and decreased 22kDa isoforms and decreased total RBP-4. Further, GHR-/- mice have increased 22kDa and decreased 19kDa isoforms. Thus, chronic GH excess and short-term GH treatment both upregulate the 19kDa isoforms of RBP-4, which can be a biomarker of GH.

A2m (one isoform) increased in GH washout group although not in GH group. This was the same isoform that was found to be increased in bGH mice. Thus, this particular isoform of a2m is a biomarker of GH. A2m is an acute phase protein, although the total a2m did not change, this isoform may play a certain role in inflammation that does not upregulate total a2m.

SAA-1 increased in GH washout group. SAA-1 was found to be decreased during normal aging in male mice (chapter III). Therefore SAA-1 is a biomarker of both aging and GH. If SAA-1 is responsive to GH, then decline over aging of SAA-1 may be due to decline to GH during aging.
Multiple Alb fragments (isoforms 1-6) all increased in GH washout group although not in GH group. GH increases Alb mRNA transcription in mice (M. Wang et al., 2008). These specific albumin isoforms are therefore long-lasting biomarkers of GH.

Together, Hp, SAA-1, specific isoform of RBP-4, a2m and Alb all could be used as markers of GH action after a relatively long washout of one week.

Regarding biomarkers of IGF-1, Hp, TTR and several Alb fragments increased. Two Hp isoforms (1 and 3), two of TTR isoforms (1 and 3), as well as six Alb isoforms (1, 2, 4, 5, 7 and 9) increased in IGF-1 group and also remained at elevated levels or even increased further in the IGF-1 washout group. The increase in Hp and Alb was in agreement with the GH groups, although in some cases different isoforms were involved. However, TTR showed an opposite change compared to GH treatment, where down-regulation was observed.

Finally, comparing the difference between GH and IGF-1 groups, apoE (isoforms 2 and 3 and total) was increased in GH but did not change in IGF-1 group; TTR isoform 3 increased in IGF-1 group but not GH group; and Alb isoforms 1, 2 and 5 increased in IGF-1 group with no change in GH group. These are biomarkers differentiating between GH and IGF-1 actions.

In general, plasma protein levels that changed by IGF-1 treatment were seen early and also lasted through the one week washout period; in contrast, in GH treated mice, protein changes usually appeared after the washout period and not acutely after the 2-week injection period, suggesting that these proteins might be regulated indirectly by GH, perhaps by IGF-1. Two exceptions are apoE and clusterin, which are altered by GH and
not IGF-1 treatment, and the change diminished during the GH washout period, suggesting that these two proteins are directly regulated by GH. In the case of TTR, apparently the negative effect of GH (as seen in bGH mice) is not immediate, and not likely mediated by IGF-1, since IGF-1 treatment increased TTR.

As mentioned earlier, IGF-1 and P-III-P are currently established as two biomarkers for GH doping. However, these molecules are not detected on our gels. Secreted form of IGF-1 is 7.7 kDa with pI 8.31 in theory. Since IGF-1 is cleaved into a and b chains like insulin, the two subunits have Mw of 3.1 and 2.5 kDa, respectively. These small sizes are beyond the limit of the gels (>8 kDa), therefore not likely detected. P-III-P is present in plasma at a very low level (~10 ng/ml (Monarca et al., 1985)), therefore not likely to be detected by 2-DE (sample loading of 2-DE is 15 ul plasma per run, resulting in 0.15ng P-III-P per gel; a spot needs to contain at least 1 ng to be detected on a 2D gel).
Conclusion

Two weeks of GH and IGF-1 injections revealed that several plasma proteins were changed by GH or IGF-1, and certain proteins were differentially changed by GH and IGF-1 (summarized in Table 12). ApoE and Clu, although altered after GH injections, did not show any difference after the washout. In contrast, the levels of other proteins such as Hp, RBP-4, SAA-1, a2m, Alb and apoA4 were different after the washout period, which represent the relative long-lasting biomarkers of GH injection.

For IGF-1, biomarkers include isoforms 1 and 3 of Hp, isoforms 1 and 3 of TTR, as well as isoforms 1, 2, 4, 5, 7 and 9 of Alb. All these proteins increased after injections and remained at higher levels after the washout period. Therefore, these proteins are biomarkers for IGF-1 doping.

Lastly, several proteins exhibit significant difference after GH and IGF-1 injections, including isoforms 2 and 3 of apoE, isoform 3 of TTR and isoforms 1, 2 and 5 of Alb. These are useful biomarkers to differentiate between GH and IGF-1. No one has to date presented plasma markers that differentiate GH from IGF-1 action. In fact, very few physiological markers exist. GH inhibits insulin action and IGF-1 has insulin-like activities such as glucose uptake, which GH does not. GH is lipolytic and IGF-1 is not. Clearly differences exist between GH and IGF-1 actions, and these are the first plasma markers that differentiate GH from IGF-1 action. For example, GH upreguates apoE and SAA-1 whereas downregulates clusterin and apoA4. All these apolipoproteins are associated with HDL (apoE also with VLDL), which suggests that GH alters cholesterol composition. None of these is altered by IGF-1, suggesting that at least for 2 weeks of
administration, IGF-1 does not alter lipid profile as much. This agrees with body compositon data where GH reduces body fat mass whereas IGF-1 does not change fat mass.

Table 12. Biomarkers of GH, IGF-1 and their difference

<table>
<thead>
<tr>
<th>Biomarkers for GH</th>
<th>Biomarkers for IGF-1</th>
<th>Differential biomarkers by GH and IGF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changed after injections</td>
<td>Changed after washout</td>
<td>Changed after injections</td>
</tr>
<tr>
<td>apoE ↑ (isoform 2 and total)</td>
<td>Hp ↑ (isoforms 1-3 and total)</td>
<td>Hp ↑ (isoforms 1 and 3)</td>
</tr>
<tr>
<td>Clu ↓ (isoforms 3-6 and total)</td>
<td>RBP-4 ↑ (one isoform of 19kDa)</td>
<td>TTR ↑ (isoforms 1 and 3)</td>
</tr>
<tr>
<td>Alb ↑ (isoforms 1-6)</td>
<td>Alb ↑ (isoforms 1, 2, 4, 5, 7 and 9)</td>
<td>Alb ↑ (isoforms 1, 2, 4, 5, 7 and 9)</td>
</tr>
<tr>
<td>A2m ↑ (one isoform)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAA-1 ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA4 ↓ (isoform 5 and total)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isoform # corresponds to Figure 65.

The above mentioned biomarkers provide a set of biomarkers for GH and IGF-1 doping in mice. Of course, extension of these results to humans is of paramount importance. A combination of these in conjunction of the already known markers of GH, such as IGF-1 and P-III-P, could provide more accurate and sensitive tests to detect GH doping.
Many biomakers are consistent between acute GH injection and bGH mice (Table 13). For example, apoE and Hp are increased by GH; whereas TTR and clusterin are decreased. In many cases, more pronounced effect is seen in bGH mice compared to the injection, perhaps due to an effect of GH dose and exposure time. For example, all five isoforms of apoE are increase in bGH mice whereas only one isoform is increased in GH injected mice. However, several proteins show difference by chronic and acute actions of GH, including albumin, MBP-C, apoA4 and SAA-1, demonstrating a differential effect of chronic versus acute actions of GH.

Table 13. Comparison of chronic and acute actions of GH on plasma proteins

<table>
<thead>
<tr>
<th>Plasma proteins</th>
<th>Chronic GH action (bGH mice)</th>
<th>Acute GH action (injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE</td>
<td>All five isoforms ↑</td>
<td>Isoform 3 and total ↑</td>
</tr>
<tr>
<td>TTR</td>
<td>8 isoforms and total ↓</td>
<td>↔ for total</td>
</tr>
<tr>
<td>Hp</td>
<td>All four isoforms ↑</td>
<td>↔ for total</td>
</tr>
<tr>
<td>Clusterin</td>
<td>Isoforms 1,2 and total ↑ (2, 4 mo)</td>
<td>Isoforms 3-6 and total ↓</td>
</tr>
<tr>
<td>RBP-4</td>
<td>Total ↓ 19kDa isoforms ↑</td>
<td>One 19kDa isoform ↑ after washout</td>
</tr>
<tr>
<td></td>
<td>22kDa isoforms ↓</td>
<td></td>
</tr>
<tr>
<td>A2m</td>
<td>Total ↔ Isoforms 2 and 3 ↑</td>
<td>Total ↔ Isoform 2↑ after washout</td>
</tr>
<tr>
<td></td>
<td>Isoforms 6 and 7 ↓</td>
<td></td>
</tr>
<tr>
<td>apoA1</td>
<td>Total and 3 isoforms ↓</td>
<td>Tend to ↑ after injection and ↓ after washout</td>
</tr>
<tr>
<td>Albumin</td>
<td>↔</td>
<td>Several isoforms ↑</td>
</tr>
<tr>
<td>MBP-C</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>apoA4</td>
<td>↔</td>
<td>Total ↔</td>
</tr>
<tr>
<td></td>
<td></td>
<td>One isoform ↓</td>
</tr>
<tr>
<td>SAA-1</td>
<td>↔</td>
<td>↑</td>
</tr>
</tbody>
</table>
CHAPTER VII. SUMMARY AND DISCUSSION OF PLASMA BIOMARKERS OF GH AND AGING

GH is a hormone with well-characterized physiological effects including promoting bone growth, protein anabolism, fat degradation, glucose output from the liver via gluconeogenesis and fluid retention (Carroll & Van den Berghe, 2001; Cheek & Hill, 1974; Clemmons et al., 1987; Fryburg & Barrett, 1993; Katzenelson, 2008; N. Moller & Jorgensen, 2009; O’Sullivan et al., 1994; Velloso, 2008; Widdowson et al.; Woodhouse et al., 2006). Elevated levels of GH is the causative factor in the pathology found in individuals with acromegaly, while lower levels of GH and GH insensitivity are associated with dwarfism displayed in GH deficient and Laron Syndrome individuals, respectively. The FDA has approved recombinant (r) hGH to treat children with the following conditions: Prader-Willi Syndrome, children born Small for Gestational Age, Turner’s Syndrome, Noonan’s Syndrome, chronic renal failure, idiopathic short stature and cystic fibrosis (Krysiak et al., 2007). In adults, rhGH has been approved to treat adult GHD (Cummings & Merriam, 1999), wasting syndrome of AIDS (Mulligan et al., 1993; Schambelan et al., 1996) and short bowel syndrome (Krysiak et al., 2007). Unfortunately, unethical doctors can prescribe rhGH for uses other than those for which it has been approved. This has led to misuse and abuse of rhGH. It has been and is abused by athletes who think that it will enhance muscle mass and physical performance. However, data suggest that GH administration has no positive role in enhancement of athletic performance (Berggren et al., 2005; Ehrnborg et al., 2005). The current biomarkers for detecting GH action for approved indications, or in a doping scenario, are not adequate.
Therefore, new biomarkers for GH action will help unravel novel target proteins regulated by GH and provide candidate markers for GH doping detection.

The GH/IGF-1 axis is involved in aging, as attenuated IGF-1 signaling over the long term leads to increased lifespan (Bonafè & Olivieri, 2009; Brown-Borg, 2009; Holzenberger et al., 2003; Tatar et al., 2003). For example, GH transgenic mice are insulin resistant and short-lived whereas GHR-/- mice are insulin sensitive and long-lived compared to WT mice. Thus, bGH mice and GHR-/- mice are two good mouse models used to study biomarkers of aging. Longitudinal analyses of plasma proteomics of these animals and their littermate controls have revealed biomarkers of chronic GH action or chronic absence of GH action as well as aging.

In this dissertation, 86 proteins have been analyzed and identified in mouse plasma using 2-DE (Figure 15). The SWISS-2DPAGE database includes 2D annotation of human plasma proteins, but not those of mice. However, several reports have been published concerning 2D PAGE reference maps for mouse plasma or serum proteins (Bijon & Jürgen, 2007, 2009; Duan et al., 2004; Gazzana & Borlak, 2008; Guipaud et al., 2007; Ren et al., 2007; Roberto et al., 2008; Skehel et al., 2000). Most of the results in this dissertation are consistent or comparable with the literature. However, a few unique findings that have not been published elsewhere are presented in this dissertation and are summarized below.

1) TTR has been identified as an ~14kDa protein as one or a few (depending on the study) isoforms (Bijon & Jürgen, 2009; Duan et al., 2004; Gazzana & Borlak, 2008;
Skehel et al., 2000). This study has identified two TTR isoforms at ~38kDa (isoforms 1 and 2 in *Figure 15A*) and as many as six isoforms in the 14kDa region (isoforms 4-9).

2) ApoE has been reported as at 34kDa protein (Bijon & Jürgen, 2009; Duan et al., 2004; Gazzana & Borlak, 2008; Skehel et al., 2000). In this dissertation, an additional isoform at 38kDa was found.

3) RBP-4 has been reported as one or two isoforms (depending on the study) at 22kDa (Bijon & Jürgen, 2009; Duan et al., 2004; Gazzana & Borlak, 2008). In this study has identified seven isoforms of RBP-4, three of ~22kDa and four of ~19kDa (isoforms 4-7) have been identified.

4) Alb fragments in the lower Mw region (<40kDa) have been reported as 1-6 isoforms (depending on the study) (Bijon & Jürgen, 2009; Duan et al., 2004; Gazzana & Borlak, 2008; Roberto et al., 2008). In this dissertation 23 alb isoforms (*Figure 15*, isoforms 1-23) have been identified, many of which have never been reported. Thus, certain isoforms of the above proteins are reported for the first time in this dissertation.

Results presented in this dissertation also have shown that several plasma proteins change significantly during aging, between bGH and WT littermates, between GHR-/- and WT littermates, between male and female mice, as well as in WT groups treated with GH or IGF-1. Discussed below are these potential biomarkers for GH action using mice injected with GH or IGF-1 as well as markers of normal aging. These proteins are shown in the 2D images in *Figure 15* of chapter III. The changes in the isoforms of these proteins are summarized in Table 14.
Table 14. Summary of protein changes during aging, and via GH action or due to gender differences

<table>
<thead>
<tr>
<th>Protein</th>
<th>Change during WT aging</th>
<th>Change in bGH vs. WT</th>
<th>Change in GHR-/- vs. WT</th>
<th>Change in GH injection</th>
<th>Change in IGF-1 injection</th>
<th>Change in female vs. male</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE</td>
<td>1-5↑, total↑</td>
<td>1,3,4 and total↓</td>
<td>3 and total↑ but ↔ washout</td>
<td>↔</td>
<td>1↑; 4↓ in female GHR-/- (similar between WT genders)</td>
<td></td>
</tr>
<tr>
<td>MBP-C</td>
<td>1↑</td>
<td>1↓(p&lt;0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2m</td>
<td>1.2↑; 5,6↓</td>
<td>2↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp</td>
<td>2,3↑</td>
<td>1-4 and total↑; 1-4 and total↑ in bGH aging</td>
<td>1-3↓(p&lt;0.05)</td>
<td>2-4 and total↑ washout</td>
<td>2,4↑</td>
<td>2↓; 3,4↓ in female WT (similar between GHR-/- genders)</td>
</tr>
<tr>
<td>Clu</td>
<td>1,2 and total↑ in bGH aging</td>
<td></td>
<td>3-6 and total↓ but ↔ washout</td>
<td>↔</td>
<td>3↓</td>
<td></td>
</tr>
<tr>
<td>RBP-4</td>
<td>2↓, 4↑, total↓</td>
<td>1-3↑, 5↓, total↑</td>
<td>6↑ washout</td>
<td>↔</td>
<td>7↑; 3,3↑; 3↓ in female aging; 1↓ in female WT (similar between GHR-/- genders)</td>
<td></td>
</tr>
<tr>
<td>TTR</td>
<td>3↑</td>
<td>1, 3-9 and total↓</td>
<td></td>
<td>6↓ washout</td>
<td>3,4↑</td>
<td></td>
</tr>
<tr>
<td>Ig kappa</td>
<td>1-3↑</td>
<td>1↑(p&lt;0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA1</td>
<td>4-6↓</td>
<td>2↓</td>
<td></td>
<td></td>
<td>2-5↑ in female aging</td>
<td></td>
</tr>
<tr>
<td>apoA4</td>
<td>3,4 and total↑</td>
<td>5↓ washout</td>
<td></td>
<td>↔</td>
<td>2,3↑ in female aging</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>6-9,18↓</td>
<td>14-17,18,20↑ washout; ↔ right after injection</td>
<td>14, 15, 17,18,21,23↑</td>
<td>19↑; 21,23↑ in female aging; 12,14-16↑ in female aging</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAA-1</td>
<td>↓</td>
<td>↑ washout</td>
<td></td>
<td>↔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prx-2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hbb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,3↓</td>
<td></td>
</tr>
</tbody>
</table>

Numbers represent isoform# of a given protein as shown in Figure 15 of chapter III. ‘Total’ is the sum of intensity of all isoforms. ↑ increase, ↓ decrease, ↔ unchanged. All changes are at a significance level of p<0.01 unless otherwise indicated (p<0.05 means 0.01<p<0.05).
ApoE is resolved as five isoforms (Figure 15). The theoretical Mw of apoE is ~34 kDa with a pI 5.46. Isoform 1 has an Mw of 38kDa, larger than the other isoforms, which are 34kDa. The identified apoE isoforms have pIs ranging from 5.76 to 6.28. The levels of all of these isoforms are increased in bGH versus WT mice from ages 2 to 19 months (Figure 34, Figure 35). Isoforms 1, 3, 4 and total apoE decreased in GHR-/- versus WT mice (Figure 52, Table 9). Isoform 3 and total apoE increased after 2 weeks of GH injection in WT mice (Figure 66). In particular, isoform 3, the most abundant apoE spot, consistently shows a significant (p<0.01) increase in bGH mice and in GH-injected mice, and decreases in GHR-/- mice. Together, these data indicate that apoE is a biomarker of GH and is positively regulated (upregulated) by GH.

The upregulation of apoE by GH has been reported in humans and rats as well (Frick et al., 2002; Johannsson et al., 1995; Jan Oscarsson et al., 1991b; Sjoberg et al., 1994a; Sjoberg et al., 1994b; Wildbrett et al., 1997) and this effect is considered to be independent of IGF-1 (Sjoberg et al., 1994b). Consistently, in our study, IGF-1 injection did not change the levels of any apoE isoforms. Unfortunately, upon GH removal, apoE returns to normal level after 7 days as shown in GH injection washout group. Therefore, in terms of window of opportunity for GH detection, apoE would not be an ideal candidate for the detection of GH doping.

ApoE also is regulated by gender. Isoform 1, the longer form, is upregulated in female mice (Table 8). Isoform 4 is decreased only in female WT mice compared to male WT and GHR-/- mice of both genders (Figure 59), suggesting an interaction of GH and gender on the level of this apoE isoform. Interestingly, the gender-specific effect on apoE
has been observed in rats such that the female pattern of GH secretion, but not male pattern, increases apoE levels (J. Oscarsson et al., 1991a; Jan Oscarsson et al., 1991b). However, our study points out that apoE may be affected by gender and/or GH in an isoform-specific way. For example, while isoform 1 is increased in females (regardless of genotype), isoform 4 is decreased in female WT mice only. Thus, without GH action (as seen in GHR-/- mice), apoE isoform 1 still is increased in females. Additionally, only the female pattern of GH secretion decreases apoE isoform 4, but not male pattern or a total lack of GH action. This suggests that in addition to gender-specific GH pattern, other gender differences also regulate apoE, in particular the PTM of apoE.

ApoE is an apolipoprotein that transports VLDL, LDL and HDL. It is also an antioxidant protein in plasma (Davignon, 2005; Miyata & Smith, 1996; Tarnus et al., 2009). Upregulation of apoE in bGH mice may be related to increased HDL and LDL cholesterol levels in these mice (Frick et al., 2001a). Moreover, decreased apoE may indicate decreased cholesterol levels in GHR-/- mice (A. Bartke et al., 2004; E. Egecioglu et al., 2005; Masternak et al., 2005a). It will be intriguing to unravel other physiological relevance of an increase in apoE in response to GH.

Three MBP-C isoforms have been identified (Figure 15). MBP-C has a theoretical Mw of ~24 kDa and pI ~5. PTMs of MBP-C include hydroxylation on proline residues and N-linked glycosylation. The three isoforms identified on 2D gels were ~32 kDa, possibly due to glycosylation and proline oxidation. Isoform 1 increases in bGH mice (Figure 36) and decreases in GHR-/- mice (p<0.05, Table 14) and therefore a biomarker of chronic GH action. MBP-C has been reported to be increased by GH treatment in
humans (Gravholt et al., 2004; T. K. Hansen et al., 2001) by increased hepatic synthesis of MBP-C (Sørensen et al., 2006). This effect is also IGF-1 independent (C. M. Sørensen, 2006; T. K. Hansen et al., 2001). However, in our study MBP-C is not altered by GH or IGF-1 injection for 2 weeks. Therefore, MBP-C is more likely a biomarker of long-term GH action and possibly longer time periods of GH treatment will result in an increase in its plasma level.

MBP-C is an innate immune defense protein against pathogens and functions to activate the classical complement pathway (Turner, 2003). Low levels of MBP-C are associated with susceptibility to frequent and chronic infections (Sumiya et al., 1991; Turner, 2003). GH has been linked to a positive modulation of innate immunity (T. K. Hansen, 2003; Kelley et al., 2007), and the increase of MBP-C in bGH mice and the decrease in GHR-/- mice may be an indicator of this action of GH.

Seven a2m isoforms with Mw ~35kDa and pI ranging from 5.65 to 7.08 have been identified (Figure 15). As discussed in chapter IV, these a2m isoforms correspond to the 35kDa subunit, which has a theoretical pI of 7.09. A2m shows isoform-specific alteration by GH. Isoforms 1 and 2 are increased while isoforms 5 and 6 are decreased in bGH versus WT mice (Table 7, Figure 44). None of the a2m isoforms are significantly altered in GHR-/- mice. Moreover, isoform 2 also increases in GH injected mice (Figure 70). Interestingly, none of the a2m isoform levels are altered by IGF-1 injection, suggesting that a2m regulation by GH is independent of IGF-1. Thus, a2m is a biomarker of GH; in particular, isoform 2 can indicate both chronic and acute GH actions. This represents a significant finding that distinguishes IGF-1 from GH actions.
Four isoforms of Hp have been identified (Figure 15). Two of the isoforms, 2 and 3, increase as WT mice age (Figure 16, Figure 21). All four isoforms are upregulated in bGH versus WT mice (Figure 37). All four isoforms also increase during aging in bGH mice at a much faster rate than in WT mice. This result positively correlates with the accelerated aging phenotype of bGH mice. Interestingly, three of the isoforms, 1-3, decrease in GHR-/- versus WT mice (p<0.05, Table 14). These have not been reported in chapter V because of not reaching a significance cutoff value of p<0.01. In both GH and IGF-1 injected mice, isoforms 2-4 increase, and remain elevated after the washout period (Figure 70, Figure 77). These data suggest that Hp is a biomarker of GH and IGF-1.

Hp is also sexually dimorphic. Isoform 2 is reduced in the plasma of female compared to male mice (Table 8). Isoforms 3 and 4 are reduced in female WT mice only, with similar levels in male WT and both genders of GHR-/- mice (Figure 59), suggesting an interaction of GH and gender on the levels of isoforms 3 and 4.

Related to Hp is Hbb, which is theoretically possesses a Mw of 15.7 kDa and a pl of 7.26. Four isoforms of Hbb have been identified in this dissertation with observed Mw ~12kDa and pls ranging from 7.24 to 7.92 (Figure 15). Isoforms 1 and 3 decrease markedly in female compared to male mice (Table 8). Hbb is a subunit of hemoglobin, which can be released into plasma due to red blood cell lysis. Incidentally, hemoglobin α-chain has been found to increase in response to GH treatment (Chung et al., 2006), but it is a different subunit (α-subunit). The presence of hemoglobin, in particular heme, imposes an oxidative stress in plasma as heme is a molecule with considerable oxidative potential (Carrell et al., 1977). Hp as an acute phase protein can be induced by such
stress. It binds to hemoglobin and quenches its oxidative damage. The lower levels of Hbb in female plasma may therefore indicate a reduced need for Hp, explaining lower levels of both Hbb and Hp in females. Hp is also an indicator of inflammation. Its induction and upregulation help to combat stress, infection and injury; however, it also reflects increased inflammation, which tends to rise as a function of age (Teunissen et al., 2003). Therefore, the higher levels of Hp in bGH mice and in mice injected with GH and IGF-1, as well as the lower levels of Hp in GHR-/- mice and the lower levels of Hbb in female mice are consistent with the healthspan and lifespan of these animals.

Clu is identified as six isoforms in this dissertation (Figure 15). These are β-subunits (see chapter IV) of Clu with Mw 34-36 kDa and pI ranging from 5.28 to 5.77. Clusterin β-chain has a theoretical Mw of 23.9 kDa and pI 6.12. Isoforms 1, 2 and total Clu show accelerated increased plasma levels as bGH mice age but do not change as WT mice age (Figure 45, Figure 46). The other isoforms (3-6) show a similar pattern although not significant at p<0.01. Plasma Clu levels are lower in bGH versus WT mice at 2 and 4 months, but quickly becomes higher than WT after 8 months, thus showing a significant interaction between GH and aging. Together with Hp, Clu also indicates the accelerated aging phenotype of bGH mice. Clu is not changed by IGF-1 injection; however, isoforms 3-6 and total Clu are decreased by GH injection (Figure 69), consistent with lower Clu levels in bGH mice at young ages (mice used for injections are 3 months of age). Thus, Clu is suppressed by GH in young mice independent of IGF-1. However, after 7 days of washout, Clu returns to normal, indicating that Clu is not a
long-lasting biomarker of GH. Clu is also sexually dimorphic. Isoform 3 decreases in female compared to male mice (Table 10).

Seven RBP-4 isoforms have been identified in this dissertation, of which isoforms 1-3 are ~22kDa and 4-7 are ~19kDa (Figure 15). RBP-4 is 21.6 kDa with pI 6 in theory. RBP-4 shows isoform-specific changes by GH action. In bGH mice, isoform 2 decreases and 4 increases (Figure 42). In GHR-/- mice, isoforms 1-3 increase and 5 decreases (Table 9). The results indicate that GH increases the 19kDa isoforms and decreases the 22kDa isoforms. Consistent with this, after the GH washout, GH injected mice have increased isoform 6, which is 19kDa (Figure 70). The regulation of RBP-4 may rely on a GH secretory pattern. Constant GH secretion as seen in females or bGH mice favors the 19kDa over 22kDa isoforms. Isoform 7 increases while isoform 3 decreases in female compared to male mice (Table 8). Isoform 1 decreases during female aging but does not change in males (Figure 50). Female WT mice have reduced isoform 1, compared to similar levels in male WT (pulsatile GH), male and in female GHR-/- mice (no GH action) (Figure 59). Therefore, high and constant GH action upregulates the 19kDa and downregulates the 22kDa RBP-4 isoforms. RBP-4 also is associated with obesity (Haider et al., 2007; Kowalska et al., 2008; Kohzo Takebayashi et al., 2007a). Consistent with these findings is the result that total RBP-4 is decreased in bGH mice (very lean) and increased in GHR-/- mice (obese).

Nine isoforms of TTR have been identified in this dissertation (Figure 15). TTR is in theory 13.6 kDa (without the secretory signal peptide) with a pI of 5.77. Among the identified TTR isoforms, 1 and 2 have Mw of ~38kDa and are likely multimers of TTR;
Isoform 3 is ~20kDa; isoforms 4-9 are probably single TTR molecules of ~14kDa. Isoform 3 increases as WT mice age (Figure 16, Figure 20). In bGH mice, except for isoform 2, all other 8 isoforms decrease compared to WT mice at all ages (Figure 40), indicating a suppression action of GH. However, this effect seems to require long-term GH action, since only isoform 6 decreases in the GH-injection/washout group (Figure 70). This suppressive effect of GH is independent of, or even antagonistic of IGF-1, since IGF-1 injection results in increased TTR isoforms 3 and 4.

Three spots have been identified as Ig kappa chain components (Figure 15). Spots 1 and 2 are Ig kappa variable region III, and spot 3 is Ig kappa constant region. All three increase as WT mice age (Figure 16) and therefore, are biomarkers of aging. Interestingly, spot 1 increases in bGH mice (p<0.05) and spot 3 decreases in GHR-/- mice (p<0.05), although not reaching a significance value of p<0.01 and therefore are not reported in chapters IV and V. Perhaps the upregulation of Ig kappa in bGH and downregulation of it in GHR-/- mice reflect their reduced and increased lifespans.

Six isoforms have been identified as apoA1 in this dissertation (Figure 15). The theoretical Mw and pI of apoA1 are ~28 kDa and ~5.4, respectively. Observed values of these isoforms were ~28 kDa and pI ranging from 5.73-6.4. Isoforms 4-6 decrease in bGH mice (Figure 41), and isoform 2 decreases in GHR-/- mice (Table 9). In this scenario, excess GH and lack of GH action both downregulate apoA1. Interestingly, while no aging effect is seen in male mice, female mice have increased apoA1 (isoforms 2-5) during aging (Figure 50), suggesting an age-related cardiovascular protective role of apoA1 specifically in females.
Five isoforms have been identified as apoA4 in this dissertation (Figure 15). ApoA4 is ~43 kDa with a pl of 5.27 in theory. The five identified isoforms are ~43 kDa with pl ranging from 5.64-5.9. Isoforms 3, 4 and total apoA4 increase in GHR-/- mice (Figure 51, Figure 52). Therefore, apoA4 is a biomarker for the lack of GH action. Isoform 2 decreases after the GH washout period (Figure 69). This isoform could serve as a biomarker for GH doping. Similar to apoA1, apoA4 also shows no change during aging in males; however, isoforms 2 and 3 of apoA4 increase during female aging. Both apoA1 and apoA4 are associated with HDL. Their levels increase as female mice age and therefore may indicate a more favorable lipid profile of females at old age.

Although full-length Alb is not resolved on our 2D gels, as many as 23 Alb fragments are detected, with Mw ranging from ~9kDa to ~33kDa (Figure 15). Isoforms 14-17, 18 and 20 increase after the GH washout period (Figure 73). Isoforms 14, 15, 17, 18, 21 and 23 increase by IGF-1 injection (Figure 79). The pattern shows that IGF-1 injection increases these isoforms relatively quickly and the increased levels are maintained after washout. However, GH treatment results in a more gradual increase in these isoforms. Thus the increase in these isoforms detected after the GH washout suggests that regulation of the levels of these Alb isoforms occur somewhat further downstream of acute GH signaling. Indeed, Alb fragments are likely the result of yet unknown proteolytic activity, which may be a target of GH and IGF-1. Although the mechanism is not clear, the fact that these Alb fragments increase after 7 days of GH washout makes them an attractive biomarker candidate for GH doping. A subset of these isoforms (#14, 15, 17 and 18) overlap with IGF-1 markers, further confirming the effect.
of GH/IGF-1 axis on the Alb isoforms. In summary, Alb isoforms 14-17, 18 and 20 are upregulated by GH; isoforms 14, 15, 17, 18, 21 and 23 are upregulated by IGF-1; and isoforms 14, 15, 17 and 18 are upregulated by both GH and IGF-1.

In addition, the Alb isoforms are also subjected to aging and gender effects. Isoforms 6-9 and 18 decrease as WT mice age (Figure 22). In WT females, isoform 19 decreases at all ages. Also, isoforms 21 and 23 increase as female mice age; isoforms 12 and 14-16 decrease as females age (Figure 50), while no change is found as males age for these isoforms. Together with results in injection studies, isoform 18 is decreased during aging, upregulated by GH and IGF-1 injections; isoform 14 and 15 are upregulated by GH and IGF-1 injections, and decreased during female aging. Thus, Alb fragmentation is regulated by GH, aging and gender.

One protein spot has been identified as SAA-1 in this dissertation (Figure 15), which is 12 kDa in theory. The observed Mw of this protein is 11.5 kDa. It decreases as WT mice age (Figure 16); therefore, it may be considered a biomarker of aging. GH injection also increases SAA-1 after the GH washout period but not immediately after GH treatment (Figure 70), suggesting that SAA-1 is a biomarker for GH doping. Further, this action is independent of IGF-1 which does not alter its level either after injection or IGF-1 washout. SAA-1 is an apolipoprotein that associates with HDL, especially during acute inflammation (Artl et al., 2000; Kisilevsky & Subrahmanyan, 1992). SAA-associated HDL (versus apoA1-associated HDL) has a larger binding affinity to macrophages, thereby mediating the uptake of HDL in macrophages. This notion has been hypothesized to lead to the transformation of macrophages into cholesterol-rich
foam cells that could deliver phospholipids and cholesterol for tissue repair during inflammation and injury (Artl et al., 2000). The foam cells are also found at atherosclerotic plaques; thus sustained inflammation may transform HDL (antiatherogenic lipoprotein particle) into a proatherogenic particle (Artl et al., 2000). Therefore, the data presented in this dissertation showing that GH injection results in increased SAA-1 may indicate an alteration of HDL during acute inflammation induced by GH.

One protein spot has been identified as Prx-2 in this dissertation (*Figure 15*). Prx-2 has a theoretical Mw of 22 kDa and pI of 5.2. On the gel, it appears to be at the location of Mw 25 kDa and pI 6.1. It decreases as WT mice age and is, therefore, a biomarker of aging (*Figure 22*). Decreased Prx-2 (an anti-oxidant enzyme) indicates a reduced capacity to fight oxidative stress as mice age.

**Biomarkers for Aging, GH and IGF-1**

In conclusion, biomarkers of aging, GH and gender have been identified in mice. Plasma biomarkers for aging include: Hp (isoforms 2 and 3), TTR (isoform 3), and Ig kappa chain (spots 1-3) that increase; and Prx-2, SAA-1 and Alb fragments (isoforms 6-9, 18) that decrease during aging in WT male mice. These are the first plasma biomarkers discovered in a longitudinal study after screening mouse plasma proteome using 2-DE. Importantly, a human cross-sectional study indicates that plasma TTR increases and Alb decreases as a function of age (Robert et al., 1999b), confirming these two proteins as biomarkers of aging. One or more of these proteins may be used as therapeutic target(s) to delay aging.
Biomarkers for chronic GH action as seen in bGH mice include apoE (all 5 isoforms), MBP-C (isoform 1), Hp (all 4 isoforms), a2m (isoforms 1 and 2) and RBP-4 (isoform 4) that increase; TTR (all isoforms except isoform 2), a2m (isoforms 5 and 6), RBP-4 (isoform 2) and apoA1 (isoforms 4-6) that decrease. These plasma proteins indicate an increased inflammatory state and a less favorable lipoprotein profile in these giant, short-lived mice (as will be discussed below), which not only provides a better understanding of the physiology of bGH mice, but also an possible explanation of their shortened lifespan.

Biomarkers for short-term GH action that last for up to 7 days after GH injection include a2m (isoform 2), Hp (isoforms 2-3), RBP-4 (isoform 6) and Alb fragments (isoforms 14-17, 18 and 20) that increase; TTR (isoform 6) and apoA4 (isoform 5) that decrease. This is the first time that novel biomarkers of short-term or acute GH treatment have been reported. Further, several of these marker proteins are the same for chronic GH action seen in bGH mice. Notably, these makers last at least until 7 days after the GH injection, at which time the body composition changes are diminished (Figure 63). This suggests that these are better markers of GH action than increased lean and decreased fat mass, the two traditional physiological activity read-outs of GH action. These plasma proteins are promising biomarkers for a novel GH doping detection. Note that some proteins do show a difference between acute and chronic GH conditions; thus similarities and disparities exist for chronic and acute GH effects on plasma proteins (Table 13 in chapter VI).
Biomarkers for short-term IGF-1 treatment that last for up to 7 days after injection include Hp (isoforms 2 and 4), TTR (isoforms 3 and 4) and Alb fragments (isoforms 14, 15, 17, 18, 21 and 23) that increase. Like GH, IGF-1 is also abused among athletes; currently there is no method to detect IGF-1 doping (Guha et al., 2009; R I G Holt, 2008). These plasma proteins therefore provide novel candidates for developing a method for IGF-1 doping detection. Note that Hp and several Alb isoforms are changed similarly in GH treatment, suggesting these are biomarkers of GH/IGF-1 actions, likely affected by IGF-1 instead of GH directly.

Importantly, biomarkers that differentiate between GH and IGF-1 actions have been reported for the first time in this dissertation, including apoE (increased by GH but not altered by IGF-1) and TTR (decreased by GH and increased by IGF-1) (Figure 80). For decades, no plasma markers are known to be differentially regulated by GH and IGF-1, although clearly the two have different functions. For example, GH inhibits insulin action and IGF-1 has insulin-like activities such as promoting glucose uptake. GH is lipolytic and anti-lipogenic whereas IGF-1 does not have these properties. These results, therefore, fills in a large gap in this area of research and hopefully will inspire further studies on the mechanisms and biological significance of the differences observed in these plasma proteins.

Biomarkers for the absence of GH action as seen in GHR-/- mice include apoA4 (isoforms 2, 4 and total) and RBP-4 (isoforms 1-3 and total) that increase; apoE (isoforms 1, 3 and 4 and total), RBP-4 (isoform 5) and apoA1 (isoform 2) that decrease. These
proteins indicate a reduced inflammatory state and favorable lipoprotein profile in this long-lived mouse.

Biomarkers for gender include apoE (isoform 1) and RBP-4 (isoform 7) that increase in females; and Hp (isoform 2), Clu (isoform 3), RBP-4 (isoform 3) and Alb fragments (isoform 19) that decrease in females compared to males. Interestingly, several proteins including apoA1, apoA4 and Alb fragments show a significant increase as females age but not in males (Table 14). Very few studies look at gender difference (Palmer et al., 2009; Serrat et al., 2007), let alone a longitudinal proteomic profile of plasma biomarkers. This result ‘opens up a new door’ for identifying gender-specific differences in proteins and how they may affect aging.

Judging from all the mouse models above, the most likely biomarkers for GH action are apoE (in particular isoform 3), Hp (in particular isoforms 2 and 3), MBP-C (isoform 1), a2m (isoform 2) and SAA-1 that increase, as well as TTR (in particular isoform 6) that decreases with GH. The best biomarkers for aging are Hp (isoforms 2 and 3) that increases and Prx-2 and SAA-1 that decrease with age.

A Model of GH’s Impact on Aging

In conclusion, I would like to present a model of aging (Figure 82) that is characterized by increased inflammation, increased cardiovascular disease risks and decreased anti-oxidative stress ability. These age-related processes are summarized in a ‘funnel’ as depicted in Figure 82. Below, I will discuss these three physiological issues in relation to the proteins discovered in this dissertation in a general way, and then more comprehensively.
Generally, inflammation is increased as normal mice age, in bGH mice and is decreased in GHR-/- mice. For example, many of the biomarkers discovered in this dissertation are positive acute phase proteins (induced and increased by inflammation), including Hp, MBP-C, SAA-1 and a2m, as well as negative acute phase proteins (acute phase protein that is decreased by inflammation) such as TTR and RBP-4 (Ananian et al., 2005; Fleck, 1989; Robert et al., 1999b). Hp increases during aging in WT mice. Hp and MBP-C are increased in bGH mice and decreased in GHR-/- mice. Increased Hp and SAA-1, as well as one isoform of a2m and the decrease in one isoform of TTR by GH injection are in line with this action of GH. On the other hand, negative acute phase proteins such as TTR and RBP-4 are decreased in bGH mice and increased in GHR-/-
mice. In terms of cardiovascular disease risks, many markers are increased during aging and in bGH mice but decreased in GHR-/- mice as manifested by several cardio-protective plasma proteins including albumin, apoA1 and apoA4. For example, albumin decreases during aging, apoA1 decreases in bGH mice and apoA4 increases in GHR-/- mice, suggesting increased cardiovascular disease risks as mice age and in bGH mice, but decreased risks in GHR-/- mice. Finally, antioxidant protein is decreased during aging. For example, Prx-2 is decreased as normal mice age, suggesting decreased ability to resist oxidative stress. Thus, increased inflammation, increased cardiovascular disease risks and decreased anti-oxidation together contribute to aging, which is promoted under chronic GH excess as seen in short-lived bGH mice, and inhibited or delayed by diminished GH/IGF-1 axis as seen in long-lived GHR-/- mice. Thus, the three physiological states shown in Figure 82 and protein ‘markers’ associated with them act together to influence mouse aging. Below, I will further expand on these issues as they relate to GH action and aging.

**Inflammation**

Inflammation is a natural defense mechanism against infection or injury. Pathogens and other immune insults activate nuclear factor (NF) κB, a transcription factor that activates a host of genes responsible for immune defense (Hayden & Ghosh, 2008). GH is known to affect the immune system. GH transgenic mice have enlarged spleen and thymus (Dialynas et al., 1999), show altered immunity, including deficiency in T helper-2 cytokine production (Gonzalo et al., 1996) and autoimmune arthritic disorders (Ogueta et al., 2000). However, the relationship between GH and inflammation is not clear. This
dissertation has identified several acute phase proteins that are upregulated in bGH mice. Acute phase proteins are induced in the liver by inflammation and are an important component of innate immune defense. GH has been shown to upregulate certain acute phase proteins such as MBP-C, TTR and RBP-4 (Gravholt et al., 2004; T. K. Hansen et al., 2001; Kemp & Canfield, 1983; Vranckx et al., 1994). It is not clear how GH induces acute phase proteins at the molecular level, although it is likely by JAK2/STAT5 pathway (Barkai et al., 2000; Dajee et al., 1996), which is also the pathway via which many cytokines are induced. GH has been shown to activate NFκB via the PI3K-Akt pathway (Jeay et al., 2002); thereby providing a potential cross-talk mechanism of GH and inflammation.

Again, the physiological role of GH in the inflammation response is not clear. Acute inflammation is featured with classic signs of redness, heat, swelling and pain. When injury or infection is cleared, these signs and symptoms of inflammation stop (Serhan & Savill, 2005). If the cause of the inflammation sustains, chronic inflammation occurs, which is featured with increased inflammatory cytokines and reactive oxygen species and eventually tissue fibrosis. Studies suggest that GH is induced under acute inflammation. For example, serum GH levels are elevated in children suffered from septic shock (de Groof et al., 2002; Önenli-Mungan et al., 2004). During sepsis caused by lipopolysaccharide (LPS), GH levels are increased (Coleman & Sartin, 1996; Lang et al., 1997). It is possible that during infection, the upregulation of GH serves as a beneficial signaling for the induction of acute phase proteins, which, together with other pro-inflammatory cytokines, are necessary to fight infection. However, GH seems to be a
double-edged sword, as high levels of GH are detrimental to critically ill patients (Y. Chen et al., 2007; Takala et al., 1999). Moreover, animals under sepsis become GH resistant with suppressed JAK2/STAT5 signaling and STAT5 DNA binding (Y. Chen et al., 2007). Thus, naturally-induced GH during infection appears beneficial; however, chronic high doses of GH treatment may have an adverse effect. Clearly in bGH mice, the increase of inflammation inferred by the plasma proteins is not due to infection or injury, but possibly chronic high levels of GH, which could induce acute phase proteins and perhaps other inflammatory cytokines such as TNF-α (unpublished observation by Valente and Berryman).

Increased inflammation is associated with aging (Brüünsgaard & Pedersen, 2003; Ferrucci et al., 2005; Roubenoff et al., 1998). Inflammatory markers such as (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 increase during aging (Kireev et al., 2009). From the results in this dissertation, it is likely that the chronic inflammation eventually leads to accelerated aging of bGH mice. There is evidence that the chronic excess of GH action causes increased oxidative stress in the liver and kidney (S. Q. Doi et al., 2000; Steven J. Hauck & Bartke, 2001). On the other hand, GHR-/- mice have reduced levels of Hp, MBP-C, indicating a reduced inflammatory state in these dwarf mice, therefore, increased lifespan (Figure 82).

Inflammation is also associated with obesity and insulin resistance (Taubes, 2009). In the case of obesity-induced insulin resistance, storage of excess fat causes enlargement of adipocytes, which attract macrophages that excrete inflammatory cytokines such as IL-1β, TNF-α and IL-6 that block insulin signaling in muscle cells via c-Jun N-terminal
kinase (JNK) that inhibits IRS-1 pathway (Figure 83). As mentioned earlier, few reports exist in literature regarding the mechanism of inflammation induced by GH. BGH mice actually have smaller adipocytes (presumably due to lipolytic action of GH); however, macrophage infiltration of adipose tissue and increased TNF-α in bGH mice have been observed (unpublished results by Valente and Berryman). Therefore, the increased inflammatory markers in these animals are not due to enlarged fat tissue; rather, excess GH may cause inflammation directly by increasing TNF-α and acute phase proteins that have been discovered in this dissertation. On the other hand, life-long suppression of GH/IGF-1 by antisense GH transgene expression in rats, a model similar to GHR-/− mice, resulted in decreased inflammation as evidenced by reduced renal infiltration of macrophages and age-related renal damage (Zha et al., 2006). In agreement with this, GHR-/− mice are protected from kidney diseases whereas bGH mice develop glomerulosclerosis prematurely (Bellush et al., 2000; T. Doi et al., 1990; T. Doi et al., 1988; Quaife et al., 1989). Interestingly, GHR-/− mice have depot-specific increases in adipocyte size compared to WT mice, with enlarged retroperitoneal and subcutaneous fat cells and unchanged epididimal adipocytes (Kelder et al., 2007). Therefore, mechanism other than fat cell size must exist to explain the altered insulin sensitivity in bGH and GHR-/− mice.
On the other hand, how excess fat is stored has also been recognized as important in terms of insulin resistance. It seems that if fat can be stored in newly formed adipocytes instead of ‘stuffing’ the existing over-sized fat cells with lipid or accumulating lipid ectopically in tissues such as liver and muscle, then insulin sensitivity can be maintained (Taubes, 2009). GHR-/- mice have reduced cell number in parametrial depot, but increased cell number in subcutaneous depot compared to controls (Flint et al., 2006), indicating that these obese mice are capable of generating more adipocytes in subcutaneous region to store the excess fat. This may be one of the reasons that explain...
their insulin sensitivity despite being fat. Interestingly, GH transgenic pigs show no mature subcutaneous adipocytes (Pinkert et al., 1994). In this aspect, it is not known if bGH mice have altered number of fat cells compared to controls. GH is known to promote adipocyte differentiation and proliferation in parametrial but not subcutaneous fat cells (Flint et al., 2006); thus GH may act on adipocytes in a depot-specific manner regarding adipocyte differentiation and proliferation. More studies are needed to uncover the action of GH in fat cell development.

Inflammation may in part explain the insulin resistance of bGH mice. GH itself is well-known to have anti-insulin actions (Cotes et al., 1949) and insulin resistance is commonly observed in acromegaly (Arya et al., 1997). GH causes insulin resistance by upregulating p85 (del Rincon et al., 2007), the regulatory subunit of PI3K, a key insulin signaling molecule. Although direct evidence is needed to link GH-induced inflammation with insulin resistance, it is likely to be another mechanism of insulin resistance by GH.

Another contributor to insulin resistance is lipotoxicity (Figure 83). In this scenario, excess fatty acids lead to accumulation of intracellular diacylglycerols (DAGs), which activate serine kinases that inhibit insulin signaling. GH transgenic mice have reduced plasma and tissues levels of triglyceride and free fatty acids, but increased cholesterol (A. Bartke et al., 2004; Frick et al., 2001a; Olsson et al., 2003; Olsson et al., 2005; Palmer et al., 2009; Zhihui Wang et al., 2007). In contrast, GHR-/- mice have reduced or unchanged plasma and tissue levels of triglyceride, free fatty acids and cholesterol (A. Bartke et al., 2004; E. Egecioglu et al., 2005; Liu et al., 2004; Masternak et al., 2005a).
Unfortunately, no data have reported DAG levels in tissues. Therefore, a role of lipid metabolism in insulin sensitivity in bGH and GHR-/- mice warrants further study.

Together, the biomarkers found in this dissertation suggests that bGH mice have increased inflammation and insulin resistance, whereas GHR-/- mice have decreased inflammation and increased insulin sensitivity, contributing to the shortened and enhanced lifespans of these mice, respectively.

Cardiovascular Disease Risk Factors

A second major physiological issue related to aging is cardiovascular disease risk factors including lipoprotein profiles. In this dissertation, it has been observed that several apolipoproteins are altered by GH. ApoE, SAA-1 are increased by GH, whereas apoA1, apoA4 and clusterin are decreased by GH. All these proteins associate with HDL (apoE also with LDL and VLDL), suggesting GH may change the protein composition of HDL. GH is known to be lipolytic and alter lipid metabolism; thus, it is not be surprising to see apolipoprotein changes in bGH and GHR-/- mice. However, the functional differences of these HDL-associated proteins warrant further study. ApoA1 is negatively associated with cardiovascular disease risks. Therefore, the decreased apoA1 may indicate an increased cardiovascular disease risk in bGH mice. On the other hand, GHR-/- mice have increased apoA4, also negatively associated with heart disease, suggesting a protective effect in these long-lived mice (Figure 82). One note of caution is that due to species difference, lipoprotein metabolism and its indication in cardiovascular disease risks may be different between humans and rodents. For example, mouse and rats have a very different lipoprotein cholesterol distribution from humans, with ~80% HDL and
~15% LDL in mouse and rat, whereas ~30% HDL and 60% LDL in humans (Fernandez & Volek, 2006). Mouse and human show important differences in apoE gene promoter region, including only a 40% DNA sequence homology and different DNA-binding protein factors (Bryan et al., 2007). Rodent models of cardiovascular diseases, heart failure and hypertrophy exhibit differences from humans in terms of myocardium action potential, cytosolic calcium exchange and heart rate (Hasenfuss, 1998). Therefore, the associations with heart disease risks for humans mentioned above are not necessarily applicable in mice.

**Oxidative Stress**

The third physiological factor related to aging is oxidative stress, which is associated with aging and considered to be the cause of aging according to some theories (Dowling & Simmons, 2009; Finkel & Holbrook, 2000; Honda & Honda, 2002). Prx-2 is an anti-oxidant enzyme that reduces reactive oxygen species. C. elegans with mutation in Prx-2 exhibits reduced lifespan (OlÅhovÅ et al., 2008). In this dissertation, Prx-2 plasma levels are found to be decreased as a function of age in WT mice, indicating a reduced capacity to fight oxidative stress at older ages (Figure 82). This result supports the oxidative stress/aging mechanism. Although the level of this protein does not show significant changes in bGH and GHR-/- mice, some evidence does show that chronic GH excess in mice causes reduced antioxidant enzymes against oxidative stress. For example, hGH mice have early declines of antioxidant enzyme superoxide dismutase (SOD) in the kidney and reduced levels of glutathione peroxidase (GPx) in the liver (Steven J. Hauck & Bartke, 2001). On the other hand, it is not clear whether GHR-/- mice possess more
antioxidant enzymes or are better at resisting stress. GHR-/- mice do not have increased antioxidant enzymes or free radical scavenge in liver and kidney compared to controls (S. J. Hauck et al., 2002). Male GHR-/- mice are less able to resist paraquat-induced stress than WT and female GHR-/- mice show no difference than WT (S. J. Hauck et al., 2002). In a more recent study, anti-oxidant enzymes are found to increase slightly (non-significant) in various tissues including liver, heart, kidney, skeletal muscle and brain of GHR-/- mice (Brown-Borg et al., 2009). Thus, more studies are needed to confirm the oxidative stress status in GHR-/- mice.

Comparing Plasma Proteins in bGH and GHR-/- Mice

A unique feature of my research was the ability to use two mouse models that display two extremes of GH signaling; giant bGH and dwarf GHR-/- mice. As presented throughout this dissertation, opposite trends of change in certain plasma proteins were observed. For example, GHR-/- mice have increased RBP-4, decreased Hp and MBP-C (p<0.05), which are the opposite in bGH mice. However, not all proteins show an opposite direction of change. For example, GHR-/- mice have normal levels of TTR, which is decreased in bGH mice. This is not entirely surprising, because if GH is involved but is not required for a process, its excess certainly produces a difference, but its absence may be rescued by other compensatory mechanisms. For example, bGH mice have elevated levels of IGF-1 protein in muscle; however, GHR-/- mice have normal levels of IGF-1 in muscle, even though circulating IGF-1 is greatly reduced (Kraemer et al., 2008). GH stimulates the immune system, however, GHR-/- mice show normal immune functions (Zhou et al., 1997), as do GH deficient Ames dwarf mice (Hall et al.,
Another example is that bGH and GHR-/- mice both have elevated levels of corticosterone compared to controls (E. Egecioglu et al., 2005; Hall et al., 2002; S. J. Hauck et al., 2001). Thus, it is not necessarily true that all biomarkers have to show opposite changes in bGH and GHR-/- mice.

Another example is that apoA4 showed normal levels in bGH mice but elevated in GHR-/- mice. As discussed in chapter V, apoA4 is an apolipoprotein transporting HDL (the ‘good’ cholesterol), and an anti-oxidant protein that removes superoxides and lipid hydroperoxide (Ostos et al., 2001; W.-M. R. Wong et al., 2007). It also has anti-atherosclerosis effect, reduces inflammation (Recalde et al., 2004), and is associated with reduced risk of cardiovascular diseases (Kronenberg et al., 2000; Warner et al., 2001). Therefore, elevated apoA4 may be an indicator of the prolonged longevity seen in GHR-/- mice (Figure 82).

Conclusion

In conclusion, this dissertation is the first proteomic study with longitudinal monitoring of plasma proteins in the entire lifespan of bGH and GHR-/- mice along with their littermate controls or normal animals. Screening of mouse plasma allows for the identification of novel biomarkers for acute, chronic and absence of GH actions. Additionally, analysis of the plasma of control (WT) mice provided the first longitudinal proteomic analysis of normal aging. The results will not only be helpful for developing a more sensitive and accurate method for detecting rhGH doping, but also have revealed several important physiological activities of GH in vivo that have not been systematically studied or explicitly proposed previously. The biomarkers for aging described in this
dissertation indicate increased inflammation and decreased anti-oxidative stress as a function of age, consistent with current theories on aging (Brüünsgaard & Pedersen, 2003; Dowling & Simmons, 2009; Ferrucci et al., 2005; Finkel & Holbrook, 2000; Honda & Honda, 2002; Roubenoff et al., 1998). Although not stressed in this write-up, these biomarkers could be potential therapeutic targets against which medical intervention may be designed.

I would like to propose that plasma biomarkers of chronic GH excess and absence are indicative of increased inflammation and unfavorable lipoprotein profiles in as seen in bGH mice, and reduced inflammation, and favorable lipoprotein profiles observed in GHR-/- mice. These two physiological states ultimately contribute to the decreased and increased lifespans observed in these two mouse models, respectively (Figure 82). Although anti-oxidative stress biomarkers decrease as a function of normal aging, a clear relationship to GH action or lack thereof was not seen. Further studies are needed to clearly show a relationship between GH and inflammation, which may ultimately unravel the mechanism of aging and the ‘fountain of youth’.
CHAPTER VIII. FUTURE DIRECTIONS

The identification of plasma biomarkers for GH and aging as discovered in this dissertation is only the first step in a long process of determining their function. Next, the source of the proteins in plasma needs to be resolved. Although most of the proteins in this dissertation are secreted by liver, some are expressed by a variety of tissues. Since isoforms of many proteins were discovered, two very important questions arise: (1) What PTM is responsible for the multiple isoforms observed on 2D gels, and (2) In what tissue does the PTM occur? As mentioned above, often one or a few specific isoforms are biomarkers of GH or aging, but not necessarily the total level of the protein. In addition, sometimes different isoforms of the same protein show opposite changes, e.g., isoforms 1 and 2 of a2m are increased in bGH mice but isoforms 5 and 6 are decreased. Unraveling the production and physiological function of the PTMs of the distinct isoforms is indeed a challenge.

Another issue would be to establish the function and mechanism of regulation of the biomarkers. For example, SAA-1 decreases with age; therefore the question becomes: what causes this decline? Similarly, since apoE is increased by GH, the outstanding question is how is it regulated and what physiological role does increased apoE play in response to GH? Understanding the mechanism responsible for the increased or decreased plasma levels of the proteins reported here is important in that it may help provide an explanation as to the mechanism by which GH acts. In a similar manner, the aging markers reported here may provide a molecule link between the aging phenotype and its underlying cause(s).
One way to study the functions of these proteins is to generate transgenic animals with overexpression or deletion of the corresponding genes encoding the proteins of interest. For example, Prx-2 decreases with aging. Thus, if a transgenic mouse overexpressing Prx-2 were demonstrated to be longer-lived, then one could propose a protective role of Prx-2 against aging. Similarly, Hp increases during aging and is upregulated in bGH mice. Thus, if an Hp transgenic mouse were shown to have accelerated aging such as seen in bGH mice, then this could confirm the causative role of Hp in promoting aging.

Knowledge on biomarkers and the mechanism of their regulation must be applied to humans. Biomarkers of rhGH action can be used to develop a novel method to detect GH doping. Likewise, plasma biomarkers of IGF-1 action would make it possible to detect IGF-1 doping. In the same way, biomarkers for aging may provide viable targets to detect precocious aging that could ultimately lead to an improvement in healthspan. Once the biomarkers are verified in humans, they may result in the generation of diagnostic kits for GH action, IGF-1 action or aging. An unanticipated result may be the discovery of therapeutic targets for abnormal GH or IGF-1 action of disorders associated with aging.

Finally, the interplay between GH action and aging is, to say the least, interesting. The lay press constantly advertises for the potential benefits of GH or GH related ‘concoctions’ in delaying the aging process. Yet, scientific evidence stresses that the lack of GH action promotes longevity. Thus, the relationship between GH action and aging is certainly controversial and stimulating. Perhaps the plasma markers that I discovered in my studies will help resolve this controversy.


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1.1 to degradation by mmp-1: Potential explanation for higher risk of type aa amyloidosis. *Rheumatology, 47*(11), 1651-1654.


WADA. (2009). The world anti-doping code, the 2009 prohibited list, international standard.


APPENDIX A: IDENTIFICATION OF PLASMA PROTEINS

Table 15. MS and MS/MS scores of proteins

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Isoform #</th>
<th>Accession #</th>
<th>Protein score b</th>
<th>Protein score C.I.% c</th>
<th>Total ion score d</th>
<th>Total ion score C.I.% c</th>
<th>Full length/start-end (of protein)</th>
<th>Start-end position of peptides matched by MS/MS (ion score, ion score C.I.% c)</th>
<th>URL link to MS/MS match sequence data</th>
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<tbody>
<tr>
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<td>1</td>
<td>P06728</td>
<td>174</td>
<td>100</td>
<td>154</td>
<td>100</td>
<td>395/21-395</td>
<td>80-97 (51, 99.607%) 317-326 (53, 99.751%)</td>
<td><a href="http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtpSz">http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtpSz</a> emT.dat&amp;hit=APOA4_MOUSE&amp;px=1&amp;ave_threshold=0.05&amp;server_mudpit_switch=0.001</td>
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<tr>
<td>apoA4</td>
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<td>P06728</td>
<td>543</td>
<td>100</td>
<td>476</td>
<td>100</td>
<td>395/21-395</td>
<td>52-65 (69, 99.994%) 80-97 (108, 100%) 155-163 (45, 98.361%) 295-305 (60, 99.954%) 317-326 (67, 99.989%) 327-345 (106, 100%)</td>
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<tr>
<td>apoA4</td>
<td>3</td>
<td>P06728</td>
<td>301</td>
<td>100</td>
<td>395/21-395</td>
<td>210-221(62, 100%)</td>
<td>295-304(68, 100%) 295-305(62, 100%) 317 - 326 (85, 100%)</td>
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<td>apoA4</td>
<td>5</td>
<td>P06728</td>
<td>147</td>
<td>100</td>
<td>395/21-395</td>
<td>52-65 (70, 100%) 80-97 (70, 100%)</td>
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<td><a href="http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtpSz">http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtpSz</a> emO.dat&amp;hit=APOA4_MOUSE&amp;px=1&amp;ave_threshold=0.05&amp;server_mudpit_switch=0.001</td>
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<td></td>
<td>Q61838</td>
<td>45</td>
<td>73.165</td>
<td>45</td>
<td>97.141</td>
<td>1495/1240-1495</td>
<td>1358-1373 (45, 97.141%)</td>
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<td>219</td>
<td>100</td>
<td>219</td>
<td>100</td>
<td>1495/1240-1495</td>
<td>1358-1373 (41, 96.875%)</td>
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<td>1418-1426 (44, 98.532%)</td>
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<td>1427-1440 (57, 99.925%)</td>
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<td>Q61838</td>
<td>293</td>
<td>100</td>
<td>293</td>
<td>100</td>
<td>1495/1240-1495</td>
<td>1307-1315 (45, 98.654%)</td>
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<td>1323-1333 (60, 99.963%)</td>
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<td>1358-1373 (48, 99.411%)</td>
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<td>1418-1426 (40, 95.654%)</td>
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<td>1427-1440 (63, 99.979%)</td>
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Clusterin (β-chain)

Clusterin (β-chain)

Clusterin (β-chain)

Clusterin (β-chain)

Clusterin (β-chain)

Clusterin (β-chain)

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http://www.matrixscience.com/cgi/protein_view.pl?file=./data/20090620/FtipSizeSm.dat&hit=THY_MOUSE&px=1&ave_thresh=34&_sigthreshold=0.05&_server_mudpit_switch=0.001
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<th>101/123 (161, 100%)</th>
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<td>124-147 (84, 100%)</td>
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<td>69-90 (77, 99.99%)</td>
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[http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtipSzE.dat&hit=TTHY_MOUSE&px=1&ave_threshold=32&_sigthreshold=0.05&_server_mudpit_switch=0.001](http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtipSzE.dat&hit=TTHY_MOUSE&px=1&ave_threshold=32&_sigthreshold=0.05&_server_mudpit_switch=0.001)

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<td>56-68</td>
<td>101-123 (91, 100%)</td>
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[http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtipSzEt.dat&hit=TTHY_MOUSE&px=1&ave_threshold=33&_sigthreshold=0.05&_server_mudpit_switch=0.001](http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20090620/FtipSzEt.dat&hit=TTHY_MOUSE&px=1&ave_threshold=33&_sigthreshold=0.05&_server_mudpit_switch=0.001)

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<th>Hbb (1 and 2)</th>
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<tr>
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</tbody>
</table>
a. Accession number in UniProtKB/Swiss-Prot database.
b. The MOWSE score is calculated by the Mascot search engine for each protein matched from the MS peak list. This score is based on the probability that peptide mass matches are non-random events. If the protein score is equal to or greater than the Mascot® Significance Level calculated for the search, the protein match is considered to be statistically non-random at the 95% confidence interval. The analysis used combined MS and MS/MS.
c. The Confidence Interval (C.I. %) for the protein score
d. A score calculated by weighting ion scores for all individual peptides matched to a given protein.
e. The C.I. % for the total ion score.
f. Total number of amino acids (in the full length protein)/position of start to end sequence (of the mature/secreted form of the protein). For example, 300/23-300 means the protein is 300 amino acids long, and the mature or secreted protein is from 23rd to 300th amino acids.
g. Start to end position of protein sequences matched by MS/MS with a C.I.%>95% except for a few. For example, 130-142 (59, 99.999%) means sequence 130th to 142nd amino acids is matched by MS/MS with a C.I.% of 99.999%.
h. Some proteins did not have good MS scores; however, their MS/MS scores were good enough to consider a confident identification. Web links to MS/MS data of these proteins are provided.
Table 16. LC/MS/MS identification of selected proteins

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<tr>
<th>Protein name</th>
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<th>Unused score</th>
<th>Total score</th>
<th>Coverage %</th>
<th>Confidence</th>
<th>Sequence e</th>
<th>Modification f</th>
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<td>4.05</td>
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<td>Gln-&gt;pyro-Glu@N-term</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>WKIDGSER</td>
<td>DSTYSMSSTLTLTKD</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DSTYSMSSTLTLTKD</td>
<td>Dethiomethyl(M)@6</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>Oxidation(M)@6</td>
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<td>DSTYSMSSTLTLTKD</td>
<td>Oxidation(M)@6</td>
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<td>Deamidated(N)@2</td>
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<td>Oxidation(W)@1</td>
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<td>99.9</td>
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<td>LPECEAVCGKPK</td>
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<td>Carbamidomethyl(C)@8</td>
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</tbody>
</table>

a. Accession number in UniProtKB/Swiss-Prot database.
b. Unused score is a measurement of all the peptide evidence for a protein that is not better explained by a higher ranking protein. It is the true indicator of protein confidence. A score of 2= 99% confidence, a score of 4=99.99% confidence, etc.
c. Total score is measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification software.

d. Sequence coverage % of the matched peptides to the full-length protein

e. Sequences matched by LC-MS/MS

f. Amino acid modification within the matched peptide in ‘sequence’ column.
<table>
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<th>Protein name</th>
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<th>Observed Mw (kDa)</th>
<th>Theoretical pI</th>
<th>Observed pI</th>
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<td>(Zanivan et al., 2008)</td>
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**Hydroxylation (proline)**

**Phosphorylation at S56,S60,T75 (H. Li *et al.*, 2009)**

**Acetylation at A2 phosphorylation**

**Phosphorylation Cleavage on pair of basic residuals**
| Albumin  | 8  | 65.9  | 23.2  | 5.53  | 6.02  |
| Albumin  | 9  | 65.9  | 23.2  | 5.53  | 6.16  |
| Albumin  | 10 | 65.9  | 23.1  | 5.53  | 6.32  |
| Albumin  | 11 | 65.9  | 16.6  | 5.53  | 5.57  |
| Albumin  | 12 | 65.9  | 16.8  | 5.53  | 5.72  |
| Albumin  | 13 | 65.9  | 16.9  | 5.53  | 5.87  |
| Albumin  | 14 | 65.9  | 16.7  | 5.53  | 6.1   |
| Albumin  | 15 | 65.9  | 16.2  | 5.53  | 6.4   |
| Albumin  | 16 | 65.9  | 16.3  | 5.53  | 6.74  |
| Albumin  | 17 | 65.9  | 16   | 5.53  | 7.12  |
| Albumin  | 18 | 65.9  | 11.1  | 5.53  | 5.62  |
| Albumin  | 19 | 65.9  | 11.3  | 5.53  | 5.78  |
| Albumin  | 20 | 65.9  | 11.6  | 5.53  | 6.2   |
| Albumin  | 21 | 65.9  | 9.1   | 5.53  | 6    |
| Albumin  | 22 | 65.9  | 9     | 5.53  | 6.34  |
| Albumin  | 23 | 65.9  | 9.2   | 5.53  | 6.6   |
| HP (α-chain) | 1 | 9.5  | 14.3  | 4.77  | 5.2   |
| HP (α-chain) | 2 | 9.5  | 14    | 4.77  | 5.4   |
| HP (α-chain) | 3 | 9.5  | 14    | 4.77  | 5.6   |
| HP (α-chain) | 4 | 9.5  | 14    | 4.77  | 5.85  |
| TTR       | 1  | 13.6  | 41.2  | 5.77  | 6.48  |
| TTR       | 2  | 13.6  | 40.1  | 5.77  | 6.85  |
| TTR       | 3  | 13.6  | 20.6  | 5.77  | 6.47  |
| TTR       | 4  | 13.6  | 14    | 5.77  | 6.08  |
| TTR       | 5  | 13.6  | 14    | 5.77  | 6.5   |
| TTR       | 6  | 13.6  | 14.3  | 5.77  | 6.66  |
| TTR       | 7  | 13.6  | 13.8  | 5.77  | 6.86  |
| TTR       | 8  | 13.6  | 13.8  | 5.77  | 7.24  |

Glycosylation on beta chain

N-Glycosylation at 118 (Bernhard et al., 2007)
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a. Unless otherwise indicated, for secreted proteins, signal peptide was removed for calculation of theoretical Mw. In case of a protein with multiple subunits, Mw of the subunit instead of the full-length protein was calculated. Mw and pI were calculated using ‘ScanSite pl/Mw’ tool from ExPaSy website ([http://scansite.mit.edu/calc_mw_pi.html](http://scansite.mit.edu/calc_mw_pi.html)). Signal peptide was determined by Swiss-Prot/TrEMBL protein database ([http://ca.expasy.org](http://ca.expasy.org)).

b. Observed Mw was an estimation from 2-D gel

c. Theoretical pI assumes no modification of the protein.

d. Observed pI was estimated from 2-D gel.

e. Unless otherwise indicated, known PTMs of proteins were reported in Swiss-Prot/TrEMBL protein database ([http://ca.expasy.org](http://ca.expasy.org)).

* Because of the heterogeneous nature of Ig kappa variable region, no theoretical Mw or pI are given.
Figure 84. Identification of a2m (isoform 4) by MS/MS. A, total ion score of MS/MS. X axis: probability based mowse score (an algorithm defined by MASCOT), y axis: number of hits (matched protein in the database). As shown, one hit (a2m) in the white
region has extensive homology to the target protein spot. B, sequences matched by MS/MS shown in red letters. Blue box represents
sequence of 35 kDa subunit of a2m; yellow box represents the signal peptide; the rest of the sequence corresponds to 165 kDa subunit
of a2m. C, individual ion scores. Ten peptides are shown. The mass errors in Da and ppm are graphed below, with each red dot
corresponding to one peptide.
APPENDIX B: ORIGINAL GEL IMAGES

Figure 85. Representative gel images of WT and bGH mice at different ages.
Figure 86. Snap shot of all 2D gels of WT mice at different ages.

Figure 87. Snap shot of all 2D gels of bGH mice at different ages.
Figure 88. Representative gel images of WT and GHR-/- mice of both genders at different ages.
Figure 89. Snap shot of all 2D gels of male WT and GHR-/- mice at different ages.

Figure 90. Snap shot of all 2D gels of female WT and GHR-/- mice at different ages.
Figure 91. Representative gel images of control, GH, IGF-1 injections and washout groups. Male mice of 3 months were used.

Figure 92. Snap shot of all 2D gels of mice in the injection study. Mice were divided into groups receiving saline, GH, IGF-1, GH then washout and IGF-1 then washout. Male mice of 3 months were used (n=7).