Depot-Specific Differences in White Adipose Tissue of Wild-Type and GHR-/- Mice of Different Ages

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Lucila Sackmann Sala
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This dissertation titled
Depot-Specific Differences in White Adipose Tissue of Wild-Type and GHR-/− Mice of Different Ages

by

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ABSTRACT

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Depot-Specific Differences in White Adipose Tissue of Wild-Type and GHR-/- Mice of Different Ages (402 pp.)

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White adipose tissue (WAT) produces many hormones and cytokines with various functions, such as glucose homeostasis, inflammation, and hemostasis, among others. The contributions of individual WAT depots to these functions differ, such that intra-abdominal depots correlate more closely than subcutaneous depots with obesity-related disorders (insulin resistance, type 2 diabetes, cardiovascular disease, etc.). However, the molecular mechanisms behind these depot-specific differences and their relation to age are not well understood. Growth hormone receptor gene-disrupted (GHR-/-) mice are dwarf and display enhanced insulin sensitivity and prolonged longevity in spite of obesity. The accumulation of WAT in GHR-/- mice is mainly in subcutaneous depots; thus, these mice represent a useful tool to study depot-specific effects on insulin responsiveness and lifespan. To analyze depot- and age-related differences in protein expression in WAT depots of GHR-/- and wild-type mice, four WAT depots (inguinal, retroperitoneal, mesenteric, and epididymal) of 12 and 24-month-old male mice of each genotype were resolved by two-dimensional gel electrophoresis and subsequent mass spectrometry. Comparisons among wild-type WAT depots showed differences in the levels of many proteins, including metabolic enzymes and stress-resistant proteins that were higher in epididymal and mesenteric depots and lower in inguinal (subcutaneous)
WAT. Also, several protein levels were consistent with higher vascularization in mesenteric WAT than in the other depots. On the other hand, age-related changes in WAT depots suggested higher aerobic glucose oxidation, lipolysis and oxidative damage at old age. Interestingly, depot- and age-related variations in protein levels were similar between wild-type and GHR-/- mice. However, genotype differences were found in the expression of proteins related to senescence and glucose uptake, among others. In conclusion, WAT depots display marked differences in their proteomes that are affected similarly with advancing age (e.g. increased insulin resistance and oxidative damage). In addition, altered senescence-related processes in WAT depots of GHR-/- mice may contribute to the favorable phenotype observed in these animals. Our results provide valuable information about WAT physiology in different depots and ages of wild-type and GHR-/- mice. Specific proteins shown to vary in this study may represent targets for treatment of age-associated insulin resistance, oxidative damage, and other WAT-related pathologies.

Approved: _____________________________________________________________

John J. Kopchick

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LIST OF ABBREVIATIONS

11β-HSD1 11β-hydroxysteroid dehydrogenase type 1
2DE two-dimensional gel electrophoresis
2PG 2-phosphoglycerate
A2M α-2 macroglobulin
ADAS cells adipose-derived stem cells
A-FABP adipocyte FABP
ALP alkaline phosphatase
ALT/GPT alanine aminotransferase
Apo apolipoprotein
ASP acylation stimulating protein
AT adipose tissue
ATGL adipose TG lipase
BAT brown AT
bGH bovine GH transgenic
BMI body mass index
CA-III carbonic anhydrase 3
CETP cholesteryl-ester transfer protein
cIAP2 cellular inhibitor of apoptosis protein 2
CK creatine kinase
CKB CK type B
CR caloric restriction
DHAP dihydroxyacetone phosphate
E-FABP epidermal FABP
Ehd2 EH domain-containing protein 2
ENO enolase
ERp29 endoplasmic reticulum resident protein 29
FABP fatty acid binding protein
FFA free fatty acids
FIRKO fat specific IR knockout
Foyo1 forkhead box protein O1
G3P glycerol-3-phosphate
G6Pase glucose-6-phosphatase
GAP glyceraldehyde-3-phosphate
G-CSF granulocyte colony stimulating factor
GGT gamma-glutamyltransferase
GH growth hormone
GHR-/- GHR/BP gene-disrupted or knockout
GHR/BP GH receptor/binding protein
GLUT4 glucose transporter 4
HMW high molecular weight
HSC70 heat shock cognate 71 kDa protein
HSL hormone-sensitive lipase
HSPβ1  heat shock protein β1
Idh3α  isocitrate dehydrogenase [NAD+] α
IEF  isoelectric focusing
IGF-1  insulin-like growth factor-1
IGFBP3  IGF-binding protein 3
IL-1  interleukin 1
IL-1ra  IL-1 receptor antagonist
IL-6  interleukin 6
IP-10  interferon γ-inducible protein 10
IPG  immobilized pH gradient
IR  insulin receptor
IRS-1  insulin receptor substrate-1
JNK1  c-Jun N-terminal kinase 1
LC  liquid chromatography
LC-CoA  long-chain coenzyme A
LPL  lipoprotein lipase
MALDI-TOF  matrix assisted laser desorption/ionization-time of flight
MAPK  mitogen-activated protein kinase
MCP-1  monocyte chemoattractant protein-1
MIP-1β  macrophage inflammatory protein-1β
MS  mass spectrometry
MSMS  tandem-MS
mTOR  mammalian target of rapamycin
MYL9  myosin regulatory light polypeptide 9
NEPHGE  nonequilibrium pH gradient electrophoresis
PAI-1  plasminogen activator inhibitor 1
p-Akt  phosphorylated Akt
p-AMPK  phosphorylated 5'-AMP-activated protein kinase
p-CREB  phosphorylated cAMP response element-binding protein
PDC  pyruvate dehydrogenase complex
PDHE1-B  pyruvate dehydrogenase E1 subunit β
PEP  phosphoenolpyruvate
PEPCK  phosphoenolpyruvate carboxykinase
PGC-1α  PPARγ coactivator 1α
PGE2  prostaglandin E2
pI  isoelectric point
PI3K  phosphatidylinositol 3-kinase
PKCζ  protein kinase Cζ
PLTP  phospholipid transfer protein
PPAR  peroxisomal proliferator activated receptor
PPLase  peptidyl-prolyl cis-trans isomerase
PRDX2  Peroxiredoxin 2
pY  tyrosine-phosphorylated
RBP4  retinol binding protein 4
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RQ</td>
<td>respiratory quotient</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>SC</td>
<td>subcutaneous</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>Shc</td>
<td>Src homology 2 domain-containing-transforming protein C1</td>
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<tr>
<td>Sod1</td>
<td>Cu/Zn superoxide dismutase</td>
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<td>TG</td>
<td>triglyceride</td>
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<tr>
<td>TIM</td>
<td>triosephosphate isomerase</td>
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<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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<td>TSH</td>
<td>thyroid stimulating hormone</td>
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<td>transthyretin</td>
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<td>TZDs</td>
<td>thiazolidinediones</td>
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<td>UCP</td>
<td>uncoupling protein</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>wild-type</td>
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CHAPTER 1: BACKGROUND

The adipose organ

For many years, adipose tissue (AT) was thought to play a passive role in the body, functioning merely as an energy store. This tissue, however, is now known to produce several hormones and cytokines that display autocrine, paracrine or endocrine functions (Flier, 1995; Hauner & Hochberg, 2002; Rajala & Scherer, 2003). Hormones and cytokines secreted by AT are termed adipokines, and bear functions involved in glucose and fatty acid metabolism, glucocorticoid and steroid hormone synthesis, and immunity, among others (Figure 1) (Kershaw & Flier, 2004; Trayhurn, 2005; Trayhurn & Wood, 2004). In addition, AT responds to endocrine regulation by classical hormones, such as insulin and growth hormone (GH), and to the central nervous system (Kershaw & Flier, 2004) (see “Regulation by…” below). Therefore, AT is now considered a true endocrine organ and a key player in the regulation of metabolism and homeostasis.

AT depots are classified into two major classes: subcutaneous (SC, under the skin) and intra-abdominal (mostly lining internal organs, also called visceral (VISC) by many authors). AT is complex at many levels, including differences among depots and the fact that adipocytes interact with surrounding cells in the tissue and with other organs (Frayn et al., 2003; Hauner & Hochberg, 2002). This points to the fact that the function of AT must be studied in a comprehensive/ integrated manner (Frayn et al., 2003). For that reason, results from research on isolated adipocytes are limited in the information they can provide, and whole AT samples are more representative of in vivo AT function.
Figure 1. Physiological and metabolic processes with which white adipose tissue is involved through the secretion of adipokines. Reproduced from Trayhurn, P. (2005), Endocrine and signalling role of adipose tissue: New perspectives on fat. *Acta Physiol Scand*, 184(4), 285-293, with permission from John Wiley and Sons.

The gene expression profile of AT has been studied in humans and rodents, but a high percentage of expressed genes (~40% in VISC and ~20% in SC fat) still have unknown function (Table 1) (reviewed by Klaus & Keijer, 2004). According to Table 1, VISC and SC fat have similar percentages of expressed genes that are involved in cell division, cell signaling, and metabolism. In contrast, the expressed genes related to cell-structure appear more predominant in SC fat, while VISC AT shows a higher percentage of expressed genes with unknown function (Klaus & Keijer, 2004). Fischer-Posovszky et al. (2007) summarized the products of known function secreted by AT, dividing them in two broad groups: those with endocrine (adiponectin, resistin, insulin-like growth factor-1 (IGF-1)/IGF-binding protein 3 (IGFBP-3), angiotensinogen, etc.) and those with
immunologic/metabolic actions (retinol binding protein 4 (RBP-4), leptin, visfatin, interleukins, plasminogen activator inhibitor 1 (PAI-1), prostaglandins, apolipoprotein E (ApoE), etc.).

Table 1

<table>
<thead>
<tr>
<th>Gene classification</th>
<th>Visceral</th>
<th>Subcutaneous</th>
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<tr>
<td>Cell division</td>
<td>6.4%</td>
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</tr>
<tr>
<td>Cell signaling/communication</td>
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<td>19.8%</td>
</tr>
<tr>
<td>Cell structure/motility</td>
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<td>12.5%</td>
</tr>
<tr>
<td>Cell/organism defense</td>
<td>5.0%</td>
<td>9.6%</td>
</tr>
<tr>
<td>Gene/protein expression</td>
<td>12.6%</td>
<td>16.9%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>14.4%</td>
<td>16.2%</td>
</tr>
<tr>
<td>Unclassified</td>
<td>39.8%</td>
<td>19.8%</td>
</tr>
</tbody>
</table>

* DNA arrays representing 16,000 cDNAs and 18,000 expressed sequence tags were used for visceral and subcutaneous tissue profiling, respectively. For the visceral depot, all genes corresponding to spots with greater than two-fold of background expression were included in the analysis (Yang YS, Song HD, Li RY, et al. The gene expression profiling of human visceral adipose tissue and its secretory functions. Biochem Biophys Res Commun 2003; 300:839) For the subcutaneous depot, only the top-ranking 300 expressed sequence tag clones (corresponding to 136 different genes) were analyzed (Gabrielsson BL, Carlsson B, Carlsson LM. Partial genome scale analysis of gene expression in human adipose tissue using DNA array. Obes Res 2000;8:374). Reprinted from Nutrition, vol. 20, Klaus, S., & Keijer, J., Gene expression profiling of adipose tissue: Individual, depot-dependent, and sex-dependent variabilities, pp. 115-120, Copyright (2004), with permission from Elsevier.
The adipose organ in mammals is composed of white and brown AT (WAT and BAT, respectively). These two tissues are morphologically and functionally different. White adipocytes are relatively large, contain elongated mitochondria with short cristae and one big central lipid droplet in the cytoplasm that “pushes” the nucleus to the periphery (Figures 3 and 4, Cinti, 2001). BAT adipocytes are smaller in size with a centrally located nucleus, abundant smooth endoplasmic reticulum, numerous and large mitochondria with several cristae, many small lipid droplets, and presence of gap junctions (Figure 5, Cinti, 2001). The high content of cytochromes in the abundant mitochondria and an increased vascularization give BAT its characteristic brown color (Klaus, 2004). Functionally, BAT is mainly used to generate heat for body temperature maintenance (Klaus, 2004), and in humans it develops mainly during gestation, and was thought to disappear after birth (Ailhaud, 2001). Recently, however, BAT was found to be present in human adult subjects, where it is hypothesized to regulate body temperature and energy expenditure (reviewed by Ravussin, 2010). WAT, on the other hand, is used mainly as an energy store (Klaus, 2004). In rats and mice, WAT cannot be detected macroscopically at birth, whereas in humans, WAT starts developing during the second trimester of the gestation period (Ailhaud et al., 1992).
Figure 2. Light microscopy of epididymal adipose tissue of a 12 days old rat. Many unilocular adipocytes are visible. Some unilocular small adipocytes are visible (arrows) along the walls of the capillary visible in the bottom. Toluidine blue-resin embedded tissue. 1,075x. Reproduced with permission from Cinti, S. (2001), Morphology of the adipose organ. In S. Klaus (Ed.), *Adipose tissue* (Vol. Chapter 2, pp. 11-26). Austin, Texas: Landes Bioscience.

Figure 3. Electron microscopy of inguinal adipose tissue (part of subcutaneous posterior depot) of a 6 days old rat. Unilocular adipocytes are visible. Inset: enlargement of the framed area. Elongated mitochondria with randomly oriented cristae are visible. Go: Golgi complex. 3,300x; inset 22,500x. Reproduced with permission from Cinti, S. (2001), Morphology of the adipose organ. In S. Klaus (Ed.), *Adipose tissue* (Vol. Chapter 2, pp. 11-26). Austin, Texas: Landes Bioscience.
Cell populations in adipose tissue

WAT is not comprised of adipocytes only, in fact, adipocytes account for one to two thirds of the total number of cells in AT (Ailhaud, 2001). The remaining cell types include preadipocytes, fibroblasts, immune cells (macrophages and mast cells), cells in vascular and neural tissue, and connective matrix (Ailhaud, 2001; Cinti, 2001; Fain et al., 2004; Kershaw & Flier, 2004) (Figure 5). Like WAT, BAT also contains other cell types, such as fibroblasts and mast cells (Cinti, 2001). When studying AT, the cell heterogeneity must be taken into consideration, because distinct cells in this tissue may be responsible for different gene expression profiles and corresponding secretion of proteins in varying amounts, resulting in unique biological activities. For this reason, as already mentioned, results from studies on isolated adipocytes do not represent the in vivo production of
proteins in AT as a whole. In fact, disregarding leptin and adiponectin, which have been found to be secreted mainly from adipocytes (Kershaw & Flier, 2004), more than 90% of the remaining adipokines are also produced in non-adipocyte cells (Fain et al., 2004). For example, levels of vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), PAI-1, and prostaglandin E2 (PGE2), are much higher in whole tissue explants than in isolated adipocytes (Fain et al., 2004).

Figure 5. Electron microscopy of epididymal adipose tissue of a 12 days old rat. In close association with two capillaries (CAP), adipocyte precursors (P) at various stages of differentiation are visible. Framed area shows an element in pericytic position displaying morphologic characteristics of a blast cell (scarce organelles and cytoplasm rich of ribosomes and polyribosomes). 6,300x. Reproduced with permission from Cinti, S. (2001), Morphology of the adipose organ. In S. Klaus (Ed.), Adipose tissue (Vol. Chapter 2, pp. 11-26). Austin, Texas: Landes Bioscience.

Individual variability among subjects is a critical factor influencing AT gene expression; even the generally considered housekeeping genes show large individual
variation in their expression levels (reviewed by Klaus & Keijer, 2004). This variability could be due to varying ratios of adipocytes/stromovascular cells among fat depots of individual subjects (Montague et al., 1997). Since many adipokines are derived primarily from non-adipocyte cell types, when analyzing gene expression and protein profiles of different adipose depots, these must be adjusted for differences in stromovascular cell proportion. Fain et al. (2004) have suggested that depot-specific differences in adipokine secretion, such as those of VEGF, IL-6 and PAI-1 (see “Regional differences…” below), may be due to a different proportion of adipocytes and non-fat cells in each anatomical location.

**Adipose tissue depots**

As stated above, WAT depots are classified into two major classes: SC and intra-abdominal. Some authors refer to all intra-abdominal depots as visceral. Others, however, use a more specific definition of VISC AT, which only includes fat depots that drain into the portal vein (mainly omental and mesenteric). The retroperitoneal, perirenal and gonadal depots, which are intra-abdominal, do not fall into this category and thus are non-VISC. In humans, when using image scans of abdominal fat (Figure 6), the division between VISC and retroperitoneal fat is delineated by the dorsal perimeter of the intestines and the ventral side of the kidneys (Wajchenberg, 2000). The more specific definition of VISC AT will be used hereinafter to avoid grouping fat depots with distinct characteristics; the fact that some intra-abdominal fat depots drain into the portal vein
suggests these fat pads bear different functions from the rest of the intra-abdominal depots.

Figure 6. Computed tomography showing cross-sectional abdominal areas at umbilicus level in two patients demonstrating variation in fat distribution. A, Visceral type (49-yr-old female, 23.1 of BMI, visceral fat area: 146 cm$^2$; subcutaneous fat area, 115 cm$^2$; V/S ratio, 1.27). B, Subcutaneous type (40-yr-old female, 24.0 of BMI, visceral fat area: 60 cm$^2$; subcutaneous fat area, 190 cm$^2$; V/S ratio, 0.31). Reproduced with permission from Wajchenberg, B. L. (2000), Subcutaneous and visceral adipose tissue: Their relation to the metabolic syndrome. *Endocr Rev*, 21(6), 697-738, Copyright 2000, The Endocrine Society. Fat areas have been colored to facilitate interpretation: visceral fat is shown in blue, subcutaneous fat in green. BMI, body mass index; V/S, visceral fat area/subcutaneous fat area. Note: there is no distinction between the different intra-abdominal fat pads; the term *visceral* here is applied to all visible intra-abdominal fat.

Cinti (2005) reviewed the anatomy of AT depots in rodents, which will be summarized here and is shown in Figure 7.

- The two main SC depots are located in the anterior and posterior parts of the body. The anterior depot, located at the base of the forelimbs, displays a central portion (interscapular) and extensions towards the front and sides. The posterior depot is divided into dorso-lumbar, inguinal, and gluteal portions, of which the inguinal portion is mainly studied. In addition, subfascial depots can be found in the limbs, at the base and the middle joint (e.g. popliteal depot in the knee).
• Thoracic depots border areas such as the heart and aorta.

• Abdominal depots include retroperitoneal (between the spine and posterior abdominal wall), perirenal (separated from the retroperitoneal depot by a peritoneal fold), omental (beside the stomach and of small size in rodents), mesenteric (lining the intestine), perigonadal (in males surrounding the epididymis, and in females the ovaries, uterus and bladder).

Figure 7. The adipose organ of an adult female Sv129 mouse maintained at 29 °C for 10 days. The organ has been dissected with the aid of a surgical microscope and each depot has been placed on the mouse profile mimicking its anatomical position. The organ is made up of two subcutaneous and several visceral depots. The most representative visceral depots are shown. Kidneys and testes were dissected together with the depots. Names of the single depot: A = deep cervical B = anterior subcutaneous (interscapular, subscapular, axillo-toracic, superficial cervical) C = visceral mediastinic D = visceral mesenteric E = visceral retroperitoneal F = visceral perirenal, peri ovaric, parametrical and perivesical G = posterior subcutaneous (dorso-lumbar, inguinal and gluteal). White areas made up of white adipose tissue and brown areas composed of brown adipose tissue are indicated by the scheme on the right. Reprinted from Prostaglandins Leukot Essent Fatty Acids, vol. 73, Cinti, S., The adipose organ, pp. 9-15, Copyright (2005), with permission from Elsevier. Note: Cinti uses the broad definition of visceral adipose tissue, including all thoracic and intra-abdominal depots in this category.
Cinti (2005) describes all adipose depots as being composed of a mixture of WAT and BAT. In adult rodents, however, only the anterior SC, mediastinac and perirenal depots show BAT macroscopically. In other depots, such as inguinal, peri-ovarian and retroperitoneal, BAT is detectable only under the light microscope and is replaced by WAT as the animal ages (Cinti, 2005).

In rodents, some fat depots have their own vascular and neural supply (anterior and posterior SC depots), whereas others use the supply from the organ to which they are connected (mesenteric from the intestine, epididymal from the testis, and perirenal from the kidney; retroperitoneal uses the parietal vascular and nerve supplies) (Cinti, 2001). Nerve fibers reach both capillaries and adipocytes in the parenchyma, although WAT is less densely vascularized and innervated than BAT (Cinti, 2005). Regarding WAT innervation, studies of catecholamine presence in whole mounts of epididymal, perirenal, inguinal and mesenteric AT from rats suggested that innervation was highest in mesenteric and lowest in inguinal fat (Rebuffé-Scrive, 1991).

Regarding embryonic development, as already stated, rat and mouse WAT cannot be detected macroscopically at birth, whereas in humans WAT starts developing during the second trimester of gestation (Ailhaud et al., 1992). Mesenchymal stem cells form clusters that give rise to WAT in the characteristic locations where it remains later in life (Ailhaud et al., 1992); at these early stages, there are no significant signs of depot-specific differences (Kiess et al., 2008). However, the origin of these mesenchymal cells is now thought to be different among facial, intra-abdominal and SC WAT (Vernochet et al., 2009). Facial WAT originates in the neural crest, whereas intra-abdominal and SC
WAT appear to derive from the sclerotome (Vernochet et al., 2009). Interestingly, progenitors of intra-abdominal and SC adipocytes present distinct developmental gene expression profiles, suggesting that distinct cell lineages might be involved in the development of these depots (reviewed by Vernochet et al., 2009).

**Regional differences in gene expression**

The complexity of WAT and the established morphologic and functional differences among fat depots are such (Klaus & Keijer, 2004) that researchers have suggested that all fat depots form a family of similar but distinctive endocrine organs (Kershaw & Flier, 2004). The importance of studying different WAT pads and performing a separate analysis for each depot has become evident through the years. The current information points mainly to SC depots behaving differently from VISC pads. In fact, in most cases VISC/ intra-abdominal (as opposed to SC) obesity correlates with cardiovascular disease, cerebrovascular disease, hypertension, glucose intolerance, type 2 diabetes, hyperlipidemia, and an overall increase in morbidity and mortality (Wajchenberg, 2000; Wajchenberg et al., 2002). However, this view has been challenged by Goodpaster et al. (1997) who found abdominal SC WAT and fat deposition in thigh muscle to correlate with insulin resistance independently of VISC fat.

Regarding lipolysis, omental WAT appears to be more responsive to activation by catecholamines and less so to inactivation by insulin than SC depots (see “Regulation…” below). Wajchenberg (2000) reviewed current data suggesting that VISC fat depots might be switched towards a more “lipolytic mode”, whereas SC fat depots would resemble a
more “lipogenic mode”. Recently, however, differences within the abdominal SC fat depot in humans have been described, with the superficial anterior region displaying higher lipolytic rates than the deep posterior one (Enevoldsen et al., 2001).

Gene expression in WAT has been studied at the mRNA (Adams et al., 2002; Atzmon et al., 2002; Dusserre et al., 2000; Lefebvre et al., 1998; X. Li et al., 2004; Montague et al., 1997; Montague et al., 1998; W. S. Yang et al., 2003) and protein (Fain et al., 2004; Fukuhara et al., 2005; Lelliott et al., 2002; X. Li et al., 2004) level both in humans and rodents. Differences in gender (Montague et al., 1997), age (Adams et al., 2002; Imbeault et al., 2000; X. Li et al., 2004), weight status (Fain et al., 2004; W. S. Yang et al., 2003), and correlation to disease states (Chiu et al., 2004; Lin et al., 2000; Masuzaki et al., 2001; Morton et al., 2004; Rahmouni et al., 2004; Tallam et al., 2005; Wallenius et al., 2002) have been analyzed. Again, these reports sometimes involve isolated adipocytes (Lefebvre et al., 1998; Lelliott et al., 2002; Montague et al., 1997; Montague et al., 1998) and other times whole tissue samples (Adams et al., 2002; Atzmon et al., 2002; Dusserre et al., 2000; Fukuhara et al., 2005; X. Li et al., 2004; W. S. Yang et al., 2003). The major differences between VISC and SC WAT gene expression and their physiological effects have been reviewed by Wajchenberg et al. (2002), including higher free fatty acid (FFA) and triglyceride (TG) turnover, and higher LPL and TG storage in VISC as opposed to SC WAT. In addition, VISC WAT displays higher local production of cortisol from cortisone, and higher angiotensinogen (promoting enhanced preadipocyte differentiation and elevation of blood pressure).
Table 2 summarizes the relative mRNA and protein levels of various products of SC and VISC WAT that are reported in the literature, as reviewed below. A matter that deserves attention when measuring mRNA or protein levels in WAT is that there are different ways to normalize the data obtained. For example, most studies measure levels of a specific product relative to total protein or total mRNA levels, or the levels of a given housekeeping gene (Table 2). However, reporting the results as relative to whole tissue weight or even per individual adipocyte are also options. Given that individual WAT depots display different weights, total mRNA/protein content, and adipocyte sizes, it follows that the results reported would vary if expressed relative to any of these variables. Possibly because other tissues/organs of the body do not present ample variability in weight and cell sizes, this matter has not received much attention in the literature. However, in the case of WAT, the normalization used should always be considered when interpreting the results. Thus, Table 2 includes information regarding the type of normalization used in the studies cited, allowing for a fair evaluation of the reported values.
<table>
<thead>
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<th>Relative to</th>
<th>T/IA/S</th>
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<th>Reference</th>
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<td></td>
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<td>Human</td>
<td>(Dusserre et al., 2000)</td>
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<td>(R. Z. Yang et al., 2006)</td>
</tr>
</tbody>
</table>

* Expressed mainly in stromovascular cells.  * Not expressed in mice. Abbreviations: GLUT4, glucose transporter 4; GS, glycogen synthase; CETP, cholesteryl-ester transfer protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCP, uncoupling protein; PAI-1, plasminogen activator inhibitor 1; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; PPAR-γ, peroxisomal proliferator activated receptor γ; TNF-α, tumor necrosis factor α; RBP4, retinol binding protein 4; IRS-1, insulin receptor substrate 1; PLTP, phospholipid transfer protein; ASP, acylation stimulating protein; VEGF, vascular endothelial growth factor; PGE2, prostaglandin E2; IL-6, interleukin 6; cIAP2, cellular inhibitor of apoptosis protein 2; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; R, mRNA; P, protein; A, activity; SP, secreted protein; SC, subcutaneous; VISC, visceral; epi, epididymal; T, whole tissue; IA, isolated adipocytes; S, isolated stromal cells.
Montague et al. (1997) measured leptin mRNA levels in isolated adipocytes from omental and abdominal SC depots from 24 human subjects. Their results showed leptin mRNA was significantly increased in SC adipocytes, particularly in women (Montague et al., 1997). Although larger adipocytes have been shown to express more leptin mRNA than smaller ones, Montague et al. (1997) suggested that the increased size of SC adipocytes could only partly account for the highly increased expression in leptin in that depot. In a later study, lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), peroxisomal proliferator activated receptor-γ (PPAR-γ), tumor necrosis factor α (TNF-α), adipsin, and RBP4 mRNA were measured, displaying no significant differences in human omental and SC adipocytes (Montague et al., 1998). Lefebvre et al. (1998) analyzed 12 human subjects for differences in abdominal SC and omental adipocytes. They assayed mRNA levels of encoded proteins involved in fatty acid metabolism, insulin signaling, and adipocyte differentiation and metabolic function (Lefebvre et al., 1998). Among the mRNA levels measured, those of GLUT4 (glucose transporter 4) and glycogen synthase were higher in SC adipocytes, whereas HSL and LPL showed no difference between depots (Lefebvre et al., 1998). Dusserre et al. (2000) used whole WAT explants from nine human subjects to measure mRNA levels of TNF-α (no difference between depots), leptin and cholesteryl-ester transfer protein (CETP) (both higher in SC fat), angiotensinogen, phospholipid transfer protein (PLTP), and acylation stimulating protein (ASP) (all higher in omental fat). W. S. Yang et al. (2003) measured adiponectin mRNA levels in fat depots of Asian women, and found a significant correlation in the expression
levels of this adipokine in the omental and the abdominal SC depots. A higher increase in adiponectin mRNA in the SC depot correlated with a smaller increase in the omental depot, suggesting that expression in the latter might be more closely regulated (W. S. Yang et al., 2003). Regarding uncoupling proteins (UCP), mRNA levels of UCP1 and UCP2 appear to be lower in SC than VISC depots in human WAT explants (Esterbauer et al., 1998; Oberkofler et al., 1998). On the other hand, gene expression of IGF-1, and IGFBP3 show no difference between those depots (Wajchenberg, 2000). A microarray study comparing gene expression in SC and perirenal fat depots of 3-month-old rats showed at least a two-fold difference in mRNA levels for ~18% of the total genes expressed in AT, many of which are related to glucose and lipid metabolism, and insulin action (Atzmon et al., 2002). Therefore, as indicated by their mRNA levels, the expression of several gene products varies among WAT depots.

It is important, however, to measure protein levels, because these may not always correlate with mRNA levels (Anderson & Seilhamer, 1997; Khochbin et al., 1991; Rousseau et al., 1992). For instance, the insulin receptor substrate-1 (IRS-1) protein levels are increased in human SC adipocytes when compared to omental adipocytes (Zierath et al., 1998), whereas the mRNA levels seem not to change between these depots (Lefebvre et al., 1998; reviewed by Vidal, 2001). Different rates of protein translation and/or turnover might account for these differences (Renes et al., 2005).
Protein levels

In addition to differences in mRNA and protein levels, there might also be differences in protein concentration and protein activity. In many cases, regulation of protein activity may involve post-translational modification, such as phosphorylation. To date, about two hundred naturally occurring post-translational modifications have been described (Creasy & Cottrell, 2004). Thus, even if the total levels of a protein remain the same, its activity might vary. HSL and perilipin are clear examples of this circumstance. Upon activation of lipolysis, both HSL and perilipin (protein that coats the lipid droplet in the adipocyte) are phosphorylated, leading to the translocation of HSL from the cytoplasm to the surface of the lipid droplet and translocation of perilipin away from the lipid droplet, allowing HSL to access the TG stores (Frayn et al., 2003). Similarly, LPL activity seems to depend on post-translational modifications, as suggested by an observed increase in activity upon feeding, which is regulated by insulin, glucocorticoids, sex steroids, and catecholamines (Wajchenberg, 2000).

Protein levels in AT have been studied by Fain et al. (2004), who found that the release of VEGF, PAI-1, PGE_2, and IL-6 was higher in human VISC than SC fat depots; and except for PAI-1, these differences were mainly originated from non-fat cells. Alessi et al. (1997) also reported higher PAI-1 secretion from VISC than SC AT. However, other studies have reported higher PAI-1 mRNA and protein secretion rates in SC than omental fat depots in humans (Eriksson et al., 2000) and no difference in basal PAI-1 mRNA levels between depots in rats (Shimomura et al., 1996). Since these studies used distinct normalization methods (Table 2), the contradictions in the data should be
interpreted with caution. The expression of the cellular inhibitor of apoptosis protein 2 (cIAP2) was studied by Li, X et al. (2004) in omental and abdominal SC fat depots, who found a significantly higher protein level in omental WAT. In rats, angiotensinogen protein secretion was lower in the SC than the epididymal fat depot (Serazin-Leroy et al., 2000). Also, LPL activity was higher in human omental isolated adipocytes than in SC ones (Marin et al., 1992a). CETP activity was similar in cultured human omental and SC AT (Shen et al., 1996). The protein CETP is another example of mRNA and protein levels being inconsistent, since, as stated previously, mRNA expression is higher in SC than omental fat. The activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) was measured in isolated stromal cells, displaying higher levels in VISC than SC depots in humans (Bujalska et al., 1997). A recently discovered adipokine, visfatin, was initially found to be increased in VISC relative to SC adipose depots in two Japanese women (Fukuhara et al., 2005). Nevertheless, a later study found no difference between visfatin mRNA in these two locations in a large sample of individuals (Berndt et al., 2005).

Protein levels of resistin are the same in omental and SC adipocytes (McTernan et al., 2002), whereas omentin is much higher in omental than SC fat (R. Z. Yang et al., 2006). Omentin is another example of an adipokine that is mainly secreted from cells in the stromovascular fraction (R. Z. Yang et al., 2006). Nevertheless, as opposed to humans, omentin was not found to be expressed in mouse WAT (SC, epididymal, retroperitoneal or mesenteric) except for low levels in perirenal fat (R. Z. Yang et al., 2006).
Other non-protein factors released or expressed mainly in WAT are estrogens and monobutyrin, and both can be found in similar levels in VISC and SC fat depots (Wajchenberg, 2000).

**Regulation by traditional hormones and the central nervous system**

As mentioned above, AT is subject to regulation by the central nervous system and by several hormones, such as glucocorticoids, sex hormones, insulin, GH, IGF-1 and thyroid hormones. The expression of receptors on the membrane of adipocytes has been characterized for some hormones better than others (Wajchenberg, 2000) and is described below.

Glucocorticoid and androgen receptors are more dense in VISC than SC depots, both in males and females (reviewed by Wajchenberg, 2000). Estrogen receptors also display differential densities among depots (Wajchenberg, 2000), and have been suggested to protect against centralization of adiposity in pre-menopausal women (Haarbo et al., 1991). On the other hand, progesterone receptors seem to be absent from human WAT (Rebuffe-Scrive et al., 1990). Insulin receptor (IR) mRNA levels are high in human omental adipocytes when compared to abdominal SC adipocytes, although omental fat is less responsive to insulin’s antilipolytic action than SC fat (Lefebvre et al., 1998). This can be explained by the high prevalence in omental adipocytes of the IR isoform A (Lefebvre et al., 1998). This isoform produces a lower activation of the downstream insulin signaling pathway than isoform B (Kosaki et al., 1995). In addition, levels of mature insulin binding receptors were reported to be similar in omental and SC
adipocytes, suggesting that not all the mRNA molecules may be translated into active proteins (Lefebvre et al., 1998).

Catecholamine receptors $\beta_1$, $\beta_2$ and $\beta_3$-adrenergic promote activation of lipolysis. $\beta_3$-adrenoceptors are mainly responsible for lipolysis in dogs and rodents, and $\beta_1$- and $\beta_2$-adrenoceptors play a major role in lipolysis in humans (Arner, 2005; Barbe et al., 1996). Additionally, humans display a fourth subtype, $\alpha_2$-adrenoceptors, which are antilipolytic (Arner, 2005). The distribution of these receptors in WAT depots shows an increased amount of $\beta$-receptors in VISC compared to abdominal SC adipocytes, with similar overall $\alpha$-responsiveness (Hellmer et al., 1992). This may explain the increased lipolytic response to catecholamines in VISC AT (Hellmer et al., 1992). Adenosine is another antilipolytic factor, apart from its action as a vasodilator. Although adenosine levels are higher in omental than SC abdominal depots, lipolytic activity of adenosine seems to be lower in the former (Vikman et al., 1991), while levels of adenosine $A_1$-receptors are low in all depots (Larrouy et al., 1991). It is important to note that in rodents these regional differences in the effects of catecholamines, insulin and adenosine are apparently not present (Arner, 2005). Nonetheless, basal lipolysis in mice, as well as humans, is higher in SC than VISC fat (Arner, 2005).

The presence of receptors for GH and thyroid hormones as it relates to density and signaling in different depots has not been fully characterized in WAT, but the role of these hormones on WAT regulation is undeniable (Wajchenberg, 2000). In rats, hypothyroidism and hyperthyroidism respectively cause transient hypo- and hyperplasia in intra-abdominal (retroperitoneal and epididymal) fat (Ailhaud et al., 1992). Regarding
GH, apart from promoting IGF-1 production, its effects on WAT include the inhibition of lipogenesis and stimulation of lipolysis. Thus, it counteracts the actions of insulin, displaying diabetogenic properties (reviewed by Kopchick & Sackmann-Sala, 2007). Studies involving different mouse models of GH action have highlighted the effects of GH on total fat content and the differential responsiveness of SC (inguinal) and intra-abdominal (epididymal and retroperitoneal) depots (Berryman et al., 2004; Berryman et al., 2006). Mice with impaired GH signaling (GH receptor/binding protein (GHR/BP) gene-disrupted or knockout (GHR-/-) mice) are dwarf and have an increased percent of body fat (see “The GHR-/- mouse” below), while mice overexpressing bovine GH (bGH transgenic mice) are giant and show a decreased percent of body fat when compared to wild-type (WT) littermates (Berryman et al., 2004; Berryman et al., 2006). When epididymal, retroperitoneal, mesenteric and inguinal pads were analyzed individually in GHR-/- mice, the increased fat mass was found to be mainly due to an augmented accumulation of fat in the inguinal (SC) depot (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010). Because, as stated above, GH is considered to counteract the action of insulin, it is interesting that the lack of GH signaling has a critical impact on SC fat depots, where insulin’s antilipolytic effects are predominant (as opposed to the VISC fat pads – mainly observed in humans, see above). This could imply that GH acts mainly as an insulin antagonist in the SC depot, and is consistent with SC fat being more insulin sensitive, in general. These hormones, however, also exert effects on adipocyte differentiation (GH), and proliferation (insulin and indirect effect of GH through IGF-1 secretion), so that the final result of a disturbed balance in their signaling cannot be easily
predicted. In fact, cell differentiating and proliferating effects of GH and insulin might be uncoupled from their lipolytic and antilipolytic effects in a depot-specific manner.

Unfortunately, most studies measuring variation in WAT mass in humans use imaging techniques for their calculations (Figure 6), without making a distinction between VISC and non-VISC WAT pads. For example, Johannsson et al. (1997) measured a decrease of 9% in total body fat, 6% in abdominal SC fat, and 18% in intra-abdominal fat after GH treatment in men with abdominal/VISC obesity using computed tomography scans. The recorded 18% reduction in intra-abdominal fat could be due to any combination of VISC and non-VISC fat pad reduction. Moreover, current reports, like the one by Attallah et al. (2006), continue to use the term visceral interchangeably with intra-abdominal, describing the effects of GH on all intra-abdominal fat pads as one.

On the other hand, while GH action on SC fat seems evident, a study by Gravholt et al. (1999) on healthy male subjects who were administered a physiological GH pulse, suggested that abdominal SC fat is more responsive to GH-induced lipolysis than the femoral SC depot. This implies that GH responsiveness varies not only among superficial and intra-abdominal AT depots, but also within SC pads of different anatomical location.

Understanding the effects of GH in WAT is complicated because GH signaling also results in the production of IGF-1, which displays insulin-like activity. In WAT, production of IGF-1 is considerable, although receptors of IGF-1 are low, probably accounting for the lower IGF-1 sensitivity in mature adipocytes as compared to skeletal muscle fibers (Yuen & Dunger, 2007). IGF-1 can signal through the IGF-1 receptor or through a hybrid insulin/IGF-1 receptor, which has 20 times more affinity for IGF-1 than
for insulin and can activate insulin-like signaling pathways (Yuen & Dunger, 2007). Thus, the described opposing actions of GH (inducing insulin resistance while promoting insulin-like action through IGF-1) make it difficult to understand GH effects on WAT. Recently, however, clinical studies in GH deficient adults, who have low IGF-1 levels, are insulin resistant and have increased VISC adiposity, have pointed out that low GH doses can accentuate the indirect (via IGF-1) effects of GH (Yuen & Dunger, 2007). Lower than standard GH doses administered to GH deficient adults produced an improvement in overall insulin sensitivity (probably by increasing IGF-1 production) with no effect on VISC adiposity, while standard GH doses induced a decrease in VISC fat mass with variable changes in insulin sensitivity (Yuen & Dunger, 2007). It seems that in humans, higher levels of GH have a larger impact on lipolysis, overriding the insulin-like activity of IGF-1, and promoting insulin resistance (Yuen & Dunger, 2007). This, however, remains to be evaluated in more detail and in other WAT depots. For instance, different results were obtained when obese type 2 diabetic mice were treated with a range of GH doses, displaying body composition changes (decreased fat mass and increased lean mass) at both low and high doses, whereas only a high dose of GH promoted an increase in circulating IGF-1 levels (List et al., 2009). Furthermore, although insulin levels remained elevated after GH administration, fasting glucose levels decreased in a dose dependent manner, following the changes in body composition (List et al., 2009). These results are difficult to compare to the study in GH deficient humans described above, given the different underlying pathologies of the sampled groups (GH deficient humans vs. obese and diabetic mice).
As described above, several physiological differences exist between SC and VISC fat, and generally, increased VISC fat (as opposed to SC fat) correlates with insulin resistance, which increases the risk for type 2 diabetes. Since VISC WAT delivers fatty acids directly to the liver through the portal vein, over the years, more attention has been paid to this adipose depot (Misra & Vikram, 2003). Nevertheless, other abdominal fat pads have been reported to influence insulin sensitivity (Table 3). Therefore, Misra & Vikram (2003) have suggested that the role of SC fat in the development of insulin resistance might be more important than currently believed.
### Table 3

*Adipose Tissue Depots Reported to Influence Insulin, Glucose, and Lipid Metabolism*

<table>
<thead>
<tr>
<th>Intra-abdominal adipose tissue</th>
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<td>Peritoneal</td>
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<td>Retroperitoneal</td>
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<table>
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<th>Subcutaneous adipose tissue*</th>
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<tbody>
<tr>
<td>Upper body</td>
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<tr>
<td>Posterior abdominal</td>
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<td>Deep abdominal</td>
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<th>Adipose tissue in the lower limbs</th>
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<tbody>
<tr>
<td>Thigh adipose tissue</td>
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<td>Subfascial</td>
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<td>Intramuscular</td>
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<th>Soleus muscle adipose tissue†</th>
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<th>Hepatic fat</th>
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Several factors related to WAT have been suggested to impair insulin sensitivity:

- FFA are the main suspects. FFA, among other lipid molecules, are thought to activate Ser/Thr protein kinases that impair insulin signaling through inhibitory phosphorylation of IRS-1 (reviewed by Kahn et al., 2006; and by Qatanani & Lazar,
Since basal lipolysis is higher in SC than VISC fat (Arner, 2005), and given that SC WAT mass is larger than VISC WAT, SC fat would be the main contributor of FFA to the bloodstream (Misra & Vikram, 2003). However, because of the closer relation of VISC WAT to the liver, FFA released from VISC fat are thought to have a bigger impact on insulin sensitivity than those from SC fat. Moreover, as stated above, lipid turnover is higher in VISC WAT, given its enhanced responsiveness to catecholamines (reviewed by Wajchenberg, 2000).

- **Adipokines that have been suggested to promote insulin sensitivity:**
  - Adiponectin is an insulin sensitizing adipokine with overall expression being higher in SC than VISC WAT (W. S. Yang et al., 2003). Thus, SC fat seems to contribute more to adiponectin-dependent insulin sensitivity than VISC fat does (Kahn et al., 2006). In obesity, however, adiponectin levels are decreased, consistent with the development of insulin resistance (Qatanani & Lazar, 2007).
  - Leptin is thought to act centrally on the control of peripheral glucose metabolism, favoring insulin sensitivity (Qatanani & Lazar, 2007). Since leptin levels are higher in SC than VISC WAT (Montague et al., 1997), increased SC fat would improve insulin sensitivity. Obesity, however, is generally characterized as a leptin-resistant state.

- **Adipokines thought to promote insulin resistance:**
  - TNF-α and RBP4 mRNA expression is similar in SC and VISC AT related to total mRNA expression (Dusserre et al., 2000; Montague et al., 1997; Montague et al., 1998).
Resistin displays similar protein levels in SC and VISC WAT per milligram of total protein (McTernan et al., 2002).

IL-6 and PAI-1 protein secretion per gram of tissue is higher in VISC than SC fat (Fain et al., 2004), although mRNA levels of PAI-1 are higher in SC than VISC fat (Eriksson et al., 2000).

To effectively compare the contribution of these five adipokines by SC and VISC WAT, however, one must consider the different total mass and protein concentration found in each fat pad. According to experimental data obtained in the laboratory from mice, total protein concentration (and probably mRNA expression) per milligram of tissue is higher in VISC (mesenteric) than SC (inguinal) fat. Taking into account that the total mass of VISC WAT is lower than that of SC WAT, the higher levels of total protein and mRNA per milligram of tissue in VISC WAT make it difficult to predict if this fat depot or the SC one might contribute the most to the total adipose-derived production of the mentioned adipokines.

Cortisol can cause insulin resistance and type 2 diabetes by counteracting insulin’s inhibition of gluconeogenesis in the liver (Qatanani & Lazar, 2007). Therefore, the activity in WAT of 11β-HSD1, which converts cortisone to cortisol, will influence insulin sensitivity. Interestingly, the activity of this enzyme has been reported to be higher in VISC than SC fat pads (Bujalska et al., 1997). Although the difference in mass of SC and VISC WAT complicates the evaluation of each depot’s contribution to the total synthesis of cortisol, VISC WAT seems critical in this case, because the effects of this glucocorticoid take place in the liver (Qatanani & Lazar, 2007).
In conclusion, according to the factors listed above, the contribution to insulin resistance and consequent type 2 diabetes by SC and VISC WAT would depend not only on adipose depot location, but also on a tight balance of depot mass, lipid turnover, and adipokine and enzyme synthesis in each WAT depot. So far, most of the literature points to a higher correlation of VISC WAT with insulin resistance. Because of its direct connection to the liver, emphasis has been applied to the relation of this type of WAT with the impairment of insulin sensitivity. However, SC WAT could also play a critical role in the development of this condition (Misra & Vikram, 2003).

**Adipocytes and lymph nodes**

Data gathered from guinea pigs by Pond and coworkers (reviewed in Pond, 1999) suggest that different adipocytes within a specific depot, although morphologically similar, may be metabolically distinct. This difference is based on the proximity of the adipocytes to the lymph nodes located in the fat depots. Paracrine interactions seem to exist, where the adipocytes near the lymph nodes have a higher responsiveness to certain cytokines (TNF-α and IL-6) than the adipocytes located further away from the lymph nodes (Pond, 1999). Probably, during local immune responses, adipocytes located near lymph nodes provide fatty acids and maybe other nutrients to the adjacent immune cells (Pond, 1999). Also, adipocytes near lymph nodes have been found to be more responsive to immune stimuli than to fasting signals (Pond, 1999). Finally, these adipocytes have a higher proportion of polyunsaturated fatty acids, which can be used by immune cells as precursors of eicosanoids and membrane components (Pond, 1999).
In this scenario, fat depots lacking lymph nodes (perirenal, gonadal, etc.) would be more homogeneous than those that contain lymph nodes (inguinal, mesenteric, retroperitoneal, etc.) (Pond, 1999).

*Pulsatility of secretion*

WAT has been reported to display some pulsatile function in terms of protein secretion. For example, leptin follows an episodic secretion pattern with short term (ultradian) pulsatility both in rodents (Figure 8) (Bagnasco et al., 2002b) and humans (Figure 9) (Licinio et al., 1998). In humans, both males and females show similar pulse frequency, with pulse amplitudes more than twice as high in females, accounting for the higher plasma leptin concentration recorded in this gender (Licinio et al., 1998). In rats, females also display higher plasma leptin levels, with higher pulse amplitudes, but slightly lower frequency than males (Bagnasco et al., 2002b). The amplitude of leptin secretion pulses decreases in both male and female rats after gonadectomy, while retaining the dimorphic pulsatility (Bagnasco et al., 2002b). Leptin pulse discharge also decreases in rats after fasting (Bagnasco et al., 2002a). Additionally, Ando et al. (2005) found circadian rhythmicity in mRNA expression of adiponectin, resistin and visfatin, but not leptin, in perigonadal fat of female mice (Figure 10).
Figure 8. Representative profiles of pulsatile leptin pattern (from 10:00 to 11:30 h) in individual male rats. Arrows in this figure indicate peaks as detected by cluster analysis. Reproduced with permission from Bagnasco, M., Kalra, P. S., & Kalra, S. P. (2002), Plasma leptin levels are pulsatile in adult rats: Effects of gonadectomy, *Neuroendocrinology*, 75(4), 257-263, Copyright 2002, S. Karger AG, Basel.

Figure 10. Daily mRNA expression profiles of adipocytokines in the visceral adipose tissue of C57BL/6J mice. Female mice were maintained under a 12-h light, 12-h dark cycle and fed a standard diet for 2 wk. Thereafter, perigonadal fat samples were obtained at Zeitgeber Time 0, 6, 12, and 18. Transcript levels of the target genes were determined by real-time quantitative RT-PCR. All data are means and SEM of five mice at each time point and are expressed as relative values to the lowest values for each gene. Reproduced with permission from Ando, H., Yanagihara, H., Hayashi, Y., Obi, Y., Tsuruoka, S., Takamura, T., et al. (2005). Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue. *Endocrinology, 146*(12), 5631-5636, Copyright 2005, The Endocrine Society. **Note:** Zeitgeber time 0 and 12 indicate the beginning of the light and dark phase, respectively.

Pulsatile secretion of FFA and glycerol have also been reported in vivo in dog (Getty et al., 2000; Hucking et al., 2003) and human SC WAT (Karpe et al., 2005) and in vitro in isolated rat adipocytes (Getty-Kaushik et al., 2005a, 2005b). This pulsatility of lipolysis is similar between dogs and rat adipocytes, ranging from seven to ten pulses per hour in frequency and five to eight minutes of pulse length, although in dogs the pulse frequency varied with time (Getty-Kaushik et al., 2005a; Getty et al., 2000; Hucking et al., 2003). Hücking et al. (2003) reported data suggesting that there are two components of lipolysis: one oscillatory, regulated by the sympathetic nervous system through β-
adrenoceptors and one constitutive, probably regulated by GH, thyroid stimulating hormone (TSH) or glucocorticoids, in times of fasting. Insulin, on the other hand, does not seem to be a primary factor in the regulation of lipolytic oscillation, because removal of insulin does not eliminate its pulsatility (Getty et al., 2000; Karpe et al., 2005). An internal pacemaker in adipocytes could also be responsible for lipolytic oscillation (Getty-Kaushik et al., 2005a, 2005b; Getty et al., 2000; Hucking et al., 2003). Studies by Getty-Kaushik et al. (2005a) in isolated rat adipocytes show pulsatility of lipolysis to be dependent on glucose and regulated by FFA. In fact, based on the fact that glucose metabolism is also oscillatory in adipocytes and that this could probably be the cause of secondary oscillations in lipolysis, Getty-Kaushik et al. (2005a) presented a model for the action of glucose on lipolysis. The model consists of oscillatory glycolysis leading to oscillatory levels of glycerol-3-phosphate (G3P), which is esterified with long-chain coenzyme A (LC-CoA) to produce TG, thus producing an oscillatory decrease in LC-CoA (Getty-Kaushik et al., 2005a). Release of inhibition of TG lipases (such as HSL) by LC-CoA would consequently be oscillatory, thus leading to bursts of lipolysis with pulsatile FFA and glycerol release (Getty-Kaushik et al., 2005a).

**Adipocyte size**

Protein expression may be affected by adipocyte size, as reported for LPL, HSL and leptin (Vidal, 2001) and many pro- and anti-inflammatory factors (Skurk et al., 2007). Skurk et al. (2007) divided cultured adipocytes into four fractions of different sizes and found a positive correlation between cell size and protein secretion and/or
mRNA expression for leptin, IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 (MCP-1), interferon γ-inducible protein 10 (IP-10), macrophage inflammatory protein-1β (MIP-1β), granulocyte colony stimulating factor (G-CSF), IL-1 receptor antagonist (IL-1ra), and adiponectin. Given that larger adipocytes seem to secrete higher amounts of the adipokines mentioned, the fact that different depots display distinct adipocyte sizes could account for depot-specific variations in the secretion of these and other proteins. Thus, mean adipocyte size should be considered as an influencing factor when analyzing depot-specific protein production.

Adipose tissue in aging

Age is an important factor affecting AT. With aging, both males and females become “fatter” as they lose fat-free mass. For instance, older subjects have a higher body fat content than younger individuals with the same body mass index (BMI) (Schwartz, 1998). Humans can lose as much as 40% of fat-free mass between the ages of 30 and 70 years, with a concomitant increase in fat content in lean tissues (such as liver, pancreas, skeletal muscle and heart) with age (Schwartz, 1998; Slawik & Vidal-Puig, 2006).

In addition, centralization of adiposity increases with age both in males and females. The rate of accumulation of intra-abdominal adiposity is gradual in males during life, whereas the rate for females is low until menopause, when central adiposity increases (Schwartz, 1998; Wajchenberg, 2000). However, there seems to be no difference in body fat distribution between 60 and 80 years of age, suggesting that the
intra-abdominal adiposity accumulation reaches a maximum around the age of 60 (Schwartz, 1998; Wajchenberg, 2000). In addition, both fat and fat-free mass usually decrease beyond the age of 85. This phenomenon is called *wasting* and generally indicates underlying disease (Miller & Wolfe, 2008).

Marin *et al.* (1992a) found that in middle-aged men the increase in intra-abdominal fat mass was explained by increased adipocyte cell sizes. Nevertheless, with further increases of central adiposity in severe obesity, the number of adipocytes was also increased (Marin et al., 1992a). Both the uptake of FFA and the responsiveness to catecholamine-induced lipolysis in middle-aged men was higher in omental than abdominal SC fat depots, suggesting that TG turnover is faster in VISC than SC WAT in older age (Marin et al., 1992a), similar to what is observed in younger individuals.

In the first few years of life, catecholamine-induced lipolysis is very low because of predominant α2-adrenoceptor action, and TSH seems to play a major role at this early age (Arner, 2005). In middle-aged and elderly individuals catecholamines have diminished effects as well, when compared to younger subjects (Arner, 2005; Imbeault et al., 2000; Lonnqvist et al., 1990). Imbeault et al. (2000) reported lower maximal lipolysis induced by a β-adrenergic agonist in abdominal and femoral SC depots of middle-aged individuals as compared to young subjects (although the β-receptor sensitivity was similar in the middle-aged and young groups). This defect in lipolysis activation in older individuals has been suggested to consist mainly of altered HSL activity (Imbeault et al., 2000; Lonnqvist et al., 1990), although new data have also shown a role for adipose TG lipase (ATGL) (Caimari et al., 2008).
In addition, the antilipolytic effect of insulin decreases in old age, at least partly due to a lower number of IRs (Arner, 2005). On the other hand, LPL activity does not seem to change with age once the increase in body fat mass is taken into account (Imbeault et al., 2000).

Another lipolytic factor that decreases with age is testosterone (Imbeault et al., 2000). Plasma testosterone levels in men correlate negatively to VISC adiposity, presumably because androgen receptor density is high in VISC fat depots, leading to high inhibition of LPL and FFA uptake (reviewed by Wajchenberg, 2000). Thus, young men with high testosterone levels display low intra-abdominal adiposity, whereas older men with lower levels of the hormone show increased intra-abdominal fat accumulation (Marin et al., 1992b). Moreover, testosterone and GH have additive effects on WAT, and GH levels also decrease at older age (S. Yang et al., 1995). Instead, in women, increased VISC adiposity is linked to elevated testosterone levels, with estrogens playing a protective role, thus delaying intra-abdominal fat accumulation until after menopause. Androgen receptors in female WAT seem to share similar characteristics to those in men, but estrogens can decrease the density of this receptor (reviewed by Wajchenberg, 2000).

The effect of physical activity on body composition of older men is different from younger subjects of similar BMI and lean body mass. After six months of endurance training, older individuals show a greater decrease in intra-abdominal WAT than younger subjects (reviewed by Wajchenberg, 2000). In a study involving middle aged parents and their offspring before and after a 12-year period, parents showed increased body fat in
spite of a decreased relative fat intake and increased exercise, suggesting an important effect of age on energy balance (Tremblay et al., 1998).

Adipocyte size and number have been studied in numerous fat depots of young and older guinea pigs (Pond et al., 1986). The adipocyte mean cell volume appears to be significantly lower in mature (>500 days) animals (sedentary, fed ad lib), with some variation among fat depots. The adipocyte number was significantly higher in animals older than 400 days in intra-abdominal VISC (omentumal and mesenteric) and non-VISC (pooled perirenal, gonadal and retroperitoneal) depots (Pond et al., 1986). This suggests that with increasing age, WAT displays more adipocytes and of smaller sizes. There was, however, no significant age dependent-change in cellularity in the abdominal superficial depot between the hind-limbs of the guinea pigs studied (Pond et al., 1986).

Finally, age-related changes in the renin-angiotensin system in WAT of rats were studied by Adams et al. (2002). They found that during the time period from four to 12 weeks of age, the expression of angiotensinogen in epididymal WAT (as well as liver) decreased, while the amount of body fat increased (Adams et al., 2002). Angiotensinogen (via angiotensin II) can increase blood pressure levels and can also act locally to promote preadipocyte differentiation.

Relationship between fat, obesity and aging

Obesity, which is defined as a syndrome caused by positive energy balance giving way to hypertrophy of WAT, is also characterized by deposition of fat ectopically in lean tissues, caused when the storage capacity of WAT is saturated (Slawik & Vidal-Puig,
The ectopic accumulation of fat associated to obesity and aging can lead to toxic effects of fat, known as lipotoxicity (Slawik & Vidal-Puig, 2006). Several features of physiological aging could lead to lipotoxicity, such as decreased energy expenditure and limited WAT expandability (Slawik & Vidal-Puig, 2006). As stated above, lean mass decreases and fat mass increases with age, and together with a characteristic decrease in physical activity and oxidative capacity, these factors can alter energy balance and increase lipid deposition (Slawik & Vidal-Puig, 2006). In obesity, the number of adipocytes increases to provide storage for the excess fat (Marin et al., 1992a), but given that this expandability of WAT seems to be limited in aging, excess fat is redirected to other organs. In addition, an age-related decrease in leptin sensitivity has been described both at the central and peripheral levels, while this adipokine has been shown to alleviate the increased accumulation of fat in the liver (Slawik & Vidal-Puig, 2006). Therefore, to some extent, the aging process may involve an inherent development of lipotoxicity; and when linked to a positive energy balance, this ectopic lipid deposition might be aggravated (Slawik & Vidal-Puig, 2006).

The relation between age, obesity, and lipotoxicity might explain why type 2 diabetes, dyslipidemia and cardiovascular disease are considered age-related diseases (Slawik & Vidal-Puig, 2006). Figure 11 illustrates the link between aging and lipotoxicity including the main factors involved, the connection established through the generation of reactive oxygen species (ROS), and the induction of apoptosis. Even in normal physiological conditions, cells suffer from imbalances between the generation of ROS through metabolism and the availability of antioxidants, resulting in the accumulation of...
oxidative damage on macromolecules such as DNA, proteins and lipids. According to the oxidative stress theory of aging, this damage leads to the progressive loss of various cellular functions as organisms age (reviewed by Muller et al., 2007).

Figure 11. Pathophysiological changes associated with lipotoxicity and aging. The interconnection of both might initiate a vicious circle accelerating development of diseases such as diabetes and heart failure. Reprinted from Ageing Res Rev, vol. 5, Slawik, M. & Vidal-Puig A.J., Lipotoxicity, overnutrition and energy metabolism in aging, pp. 144-164, Copyright (2006), with permission from Elsevier.

On a different level, reduced WAT mass, especially VISC, has been associated with increased lifespan, either by caloric restriction (CR) or by mutations affecting insulin signaling (reviewed by Kloting & Bluher, 2005). Moreover, a common finding in centenarian subjects is higher insulin sensitivity and lower fat content than younger subjects (Kloting & Bluher, 2005). Alterations in nutrient-sensing pathways and insulin/IGF-1 signaling have been largely related to longevity extension in yeast and many animal models (reviewed by Fontana et al., 2010). However, surgical removal of VISC WAT by itself has also been shown to increase mean and maximum lifespan in rats.
(Muzumdar et al., 2008). On the other hand, lipodystrophy, a condition displaying low fat mass, is associated with low insulin sensitivity and shorter lifespan, revealing that low fat content per se is not the cause of increased lifespan (Kloting & Bluher, 2005). In fact, it might be the location of WAT accumulation that influences longevity. The protein SIRT1 has been proposed as the link between low adiposity and increased lifespan, but there is currently no experimental evidence to support this (Kloting & Bluher, 2005). SIRT1 is a NAD$^+$-dependent deacetylase that modulates cellular metabolism based on nutritional status and has been suggested to mediate at least part of the longevity extending effects of CR (reviewed by Haigis & Sinclair; and by Yu & Auwerx, 2009).

Preadipocytes and senescence

In humans, rats and mice, the preadipocyte population in WAT has been shown to display inherent depot-specific differences (reviewed by Sepe et al., 2010). These findings highlight the importance of analyzing each WAT depot separately, especially given that preadipocytes represent 15-50% of cells in WAT (Kirkland et al., 1994). Moreover, because WAT turns over throughout life, preadipocyte properties may dictate the characteristics of adipocytes in each WAT depot and their behavior in aging (Cartwright et al., 2010). Depot-specific preadipocyte properties include their differentiation capacity, which in humans is highest in SC preadipocytes, intermediate in mesenteric and lowest in omental cells (Tchkonia et al., 2007). Interestingly, even though both mesenteric and omental WAT are VISC depots (both depots drain into the portal vein), the gene expression profiles of mesenteric preadipocytes are more similar to SC
than omental cells (Tchkonia et al., 2007). In rats, replication capacity is higher in retroperitoneal than epididymal preadipocytes and decreases with age in both depots (with a more marked decline in retroperitoneal WAT) (Kirkland et al., 1990). Consistent with this, age-related changes in preadipocyte gene expression were found to be greater in retroperitoneal than epididymal WAT, including increased expression of genes involved in cell stress and decreased expression of genes that regulate differentiation (Cartwright et al., 2010). In fact, the redistribution of body fat from SC to VISC depots in aging might reflect an exhausted capacity of replication of preadipocytes in SC WAT, resulting in decreased differentiating capacity in this depot compared to VISC WAT (reviewed by Cartwright et al., 2007). A progressive accumulation of senescent preadipocytes that takes place at different rates in each WAT depot has been suggested to be responsible for the fat redistribution that takes place in aging (Cartwright et al., 2010).

The GHR-/– mouse

The disruption of the GHR/BP gene in mice was generated in our laboratory by homologous recombination at the level of the gene’s fourth exon in 129 Ola-derived embryonic stem cells injected into BALB/cJ blastocysts (Zhou et al., 1997). The heterozygous GHR+/- Ola-BALB/cJ male animals obtained, were backcrossed to WT C57BL/6J females for eight generations, to obtain 99.61% congenic mice (Coschigano et al., 2003). The line was maintained thereof by crossing GHR+/- males to GHR+/- females. Because most, if not all, GH actions are carried out via the GHR, GHR-/- mice

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1 In the works cited, the retroperitoneal depot is called perirenal, but conversations maintained with Dr. Kirkland and members of our laboratory have established that the WAT depot studied is actually the retroperitoneal one.
have impaired GH signaling, which makes them a valuable tool for the study of GH action.

Phenotypic characteristics of GHR-/- mice have been studied and reported in over 100 publications (List et al., 2010). As already mentioned, GHR-/- mice are dwarf (Figure 12), with significantly decreased growth rate, body length and weight (Coschigano et al., 2003). All organs show reduced weight in proportion to body size, except brain and AT, which show increased, and liver and kidney, which show decreased weight when normalized to body size (Berryman et al., 2006). As a result of impaired GH signaling, plasma IGF-1 levels are low (20% of normal) (Coschigano et al., 2003). IGFBP levels also are affected: significantly lower IGFBP1, 3 and 4 and higher IGFBP2 levels are detected in serum of GHR-/- mice when compared to control littermates (Coschigano et al., 2003).

*Figure 12. Wild type (left) and GHR-/- mouse (right) of the C57BL/6J genetic background, size differences can be easily observed.*
One of the major characteristics of GHR-/- mice is their significantly increased lifespan (26% longer than WT); WT male mice of the C57BL/6J background live an average of ~760 days (~2 years) and GHR-/- mice reach an average of ~950 days (~7 months more than controls) (Coschigano et al., 2003). Studies on sexual maturation of GHR-/- mice suggest that their increased lifespan is due to slower overall aging, instead of an extended senescence (reviewed by List et al., 2001; List et al., 2010).

Adipose tissue, body composition and energy balance in GHR-/- mice

GHR-/- mice display enhanced accumulation of AT; this is consistent with the lack of GH’s lipolytic action and increased insulin activity leading to lipogenesis. Relative to body weight, food consumption in GHR-/- mice is significantly higher than control mice (Berryman et al., 2006; Coschigano et al., 2003; Egecioglu et al., 2006; Longo et al., 2010) or shows a trend towards increase (Berryman et al., 2004; Coschigano et al., 2003). Metabolic rate, as estimated by oxygen consumption, is also higher in GHR-/- than control mice when expressed relative to whole body mass or lean body mass (Berryman et al., 2006; Longo et al., 2010; Westbrook et al., 2009). GHR-/- mice also display lower respiratory quotient (RQ) than controls, which indicates a preference to oxidize lipids over carbohydrates (Longo et al., 2010; Westbrook et al., 2009). As already mentioned, percent body fat is significantly higher in male GHR-/- than control mice; this has been observed as early as six weeks of age and for the duration of their lifespan (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Bonkowski et al., 2006a; Egecioglu et al., 2006). Females follow the same trend but the difference from
WT is not as marked (Berryman et al., 2010; Bonkowski et al., 2006a). Notably, the accumulation of fat mass in GHR-/- mice is not uniform and displays regional differences for distinct AT depots. The SC fat depot is markedly increased in both male and female GHR-/- mice when normalized to body weight at all ages examined (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Flint et al., 2006; Y. Li et al., 2003; Liu et al., 2004). Other depots display variable results; the retroperitoneal one, for example, shows increased weight per gram of body weight in GHR-/- mice (Berryman et al., 2004; Egecioglu et al., 2006) or no significant change from WT values (Berryman et al., 2006; Berryman et al., 2010; Liu et al., 2004). Gonadal (epididymal or parametrial) adipose depots show no change between GHR-/- and control mice after normalization to body weight (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Egecioglu et al., 2006; Y. Li et al., 2003) or a significant decrease in GHR-/- mice (Coschigano et al., 2003; Flint et al., 2006). Mesenteric fat has only been reported by Berryman et al. (2010) and displayed no changes after normalization to body weight in 2-year old GHR-/- mice compared to WT. Regarding BAT, an increase in interscapular BAT mass relative to body weight is observed in GHR-/- mice when compared to controls (Egecioglu et al., 2006; Y. Li et al., 2003). This depot also displays increased expression of UCP-1 in GHR-/- than WT animals (Y. Li et al., 2003). Fat depot weights in GHR-/- mice as compared to WT have been summarized in Table 4.
Table 4

Relative Mass of AT Depots Normalized to Body Weight in GHR-/- Mice Compared to WT Mice

<table>
<thead>
<tr>
<th>Age (months)</th>
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<th>Retro M</th>
<th>F</th>
<th>Gon M</th>
<th>F</th>
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</table>

Arrows indicate the direction of change (increase ↑, decrease ↓, no change ↔) for the levels measured in GHR-/- mice as compared to WT. Abbreviations: SC, subcutaneous; Retro, retroperitoneal; Gon, gonadal (epididymal or parametrial); Mes, mesenteric; BAT, interscapular BAT; M, male; F, female. BAT has not been measured in female mice.

Given that GH affects adipocyte differentiation and metabolism, mean adipocyte size has been measured in our laboratory in retroperitoneal, epididymal and inguinal WAT of 6-month-old male GHR-/- and control mice, and an increase was found in GHR-/- retroperitoneal (11%) and inguinal (33%) mean adipocyte size, relative to controls (Kelder et al., 2007). In the same study, SC adipocytes of control mice were found to be smaller than those from control intra-abdominal depots. On the other hand, Flint et al. (2006) found adipocyte numbers to be increased in the SC depot of female GHR-/- mice when compared to controls. Interestingly, preadipocytes from this depot showed normal response to insulin or isoproterenol (β-adrenoreceptor agonist) in GHR-/- mice, whereas preadipocytes from the parametrial depot displayed lower proliferation.
and differentiation capabilities (Flint et al., 2006). It follows that the absence of GHR produces distinct effects in preadipocytes of each adipose depot, which agrees with the vast differences in depot sizes measured in GHR-/- mice. The molecular mechanisms behind these effects are certainly intriguing.

Metabolism and metabolic regulation of the GHR-/- mouse

Given their increased lifespan and enhanced insulin sensitivity in spite of obesity, the metabolic regulation of GHR-/- mice has been the target of numerous studies. In addition to changes in the GH/IGF-1 axis, GHR-/- mice show decreased blood glucose at young ages that seems to normalize as the animals become older (Al-Regaiey et al., 2005; Coschigano et al., 2003). Because GH is well known to display diabetogenic properties, it is not surprising to find enhanced insulin sensitivity in GHR-/- mice.

In fact, their insulin levels are very low throughout their lifespan (Al-Regaiey et al., 2005; Coschigano et al., 2003).

Blood chemistry profiles of the GHR-/- mouse

Glucose and insulin

Fasting and non-fasting glucose levels are significantly lower in young male and female (<10-month-old) GHR-/- mice compared to WT controls (Bartke et al., 2004; Berryman et al., 2006; Coschigano et al., 2003; Dominici et al., 2000; Egecioglu et al., 2006; Hauck et al., 2001; Liu et al., 2004; Panici et al., 2009). However, glucose levels

\footnote{Most of the text in this section (word for word) has been submitted for publication to \textit{Endocrine Reviews} (List et al., 2010) and some parts will be published in Sackmann-Sala et al. (in press, reproduced with kind permission of Springer Science+Business Media).}
increase in GHR-/- males at older ages, becoming similar to those of WT animals (Al-Regaiey et al., 2005; Bonkowski et al., 2009; Coschigano et al., 2003; Panici et al., 2009). Unfortunately, glucose levels of older GHR-/- females have not been reported.

Insulin levels are extremely low in GHR-/- mice and, unlike glucose, remain significantly lower than WT controls for their entire lifespans (Al-Regaiey et al., 2005; Berryman et al., 2006; Bonkowski et al., 2009; Coschigano et al., 2003; Dominici et al., 2000; Egecioğlu et al., 2006; Guo et al., 2005; Hauck et al., 2001; Liu et al., 2004; Panici et al., 2009). Therefore, GHR-/- have increased insulin sensitivity (Bonkowski et al., 2009; Bonkowski et al., 2006b; Guo et al., 2005; Liu et al., 2004; Masternak et al., 2005a; Panici et al., 2009), which, as already explained, is consistent with their extended longevity (reviewed by Fontana et al., 2010).

Glucagon

In a similar age-dependent pattern as glucose, circulating glucagon levels in GHR-/- mice are lower than WT at two months of age (Liu et al., 2004) and not different than WT by 21 months (Al-Regaiey et al., 2005). In addition, after a glucose load, the decrease in glucagon levels is more marked in GHR-/- than WT mice at two to four months of age (Guo et al., 2005). Given that glucagon stimulates glucose production in the liver, it fits that circulating glucose levels show the same trend as this hormone.

Glucocorticoids

Glucocorticoids are elevated in young and old male GHR-/- mice but not in young females (Al-Regaiey et al., 2005; Egecioğlu et al., 2006; Hauck et al., 2001); unfortunately, no measurements of corticosterone have been reported for older females.
Consistent with higher corticosterone levels, expression of the glucocorticoid activating enzyme 11β-HSD1 is higher in livers of young male GHR-/− than WT mice. Together with low insulin, increased corticosterone levels are consistent with the activation of gluconeogenesis in the liver. In fact, increased levels of several gluconeogenic enzymes have been found in the livers of GHR-/− mice (Al-Regaiey et al., 2005) (see “Insulin signaling” below).

**Adipokines**

Blood levels of other hormones related to glucose homeostasis have also been reported, such as leptin and adiponectin. As mentioned previously, these hormones are secreted by WAT and have known insulin-sensitizing actions. In GHR-/− mice, circulating leptin levels are increased in non-fasted ~5 and 21-month-old males (Al-Regaiey et al., 2005; Egecioglu et al., 2006). However, in 5-month-old GHR-/− mice, leptin levels are normal after a 4-hour fast (Berryman et al., 2004). On the other hand, circulating adiponectin levels are increased in ~6 and 21-month-old males, and ~5-month-old female GHR-/− mice (Al-Regaiey et al., 2005; Berryman et al., 2004; Nilsson et al., 2005). In addition, high molecular weight (HMW) adiponectin, which is thought to represent the active form of this adipokine (Kaser et al., 2008; Waki et al., 2003; Whitehead et al., 2006), and the ratio of HMW to total adiponectin were both higher in 6-month-old male GHR-/− mice than controls (Lubbers et al., unpublished results). Interestingly, in this mouse model, high adiponectin levels are associated with obesity; it appears that insulin sensitivity is a stronger factor than obesity in the correlation to
adiponectin levels. Alternatively, the HMW form of adiponectin might be more closely related with insulin sensitivity than with body fat mass.

Lipid profiles

Regarding lipid profiles, circulating cholesterol levels are generally lower in GHR-/- than WT mice. In males, total cholesterol levels are lower than WT at young and old age (Bartke et al., 2004; Egecioglu et al., 2006; Masternak et al., 2005a). However, when data from male and female GHR-/- mice are combined, total cholesterol levels are not different between genotypes (Liu et al., 2004). Unfortunately, no data are available on cholesterol levels of females alone. In young males, HDL-cholesterol is also lower in GHR-/- than WT mice (Egecioglu et al., 2006), and grouped male and female GHR-/- mice show a trend towards lower HDL-cholesterol than WT animals (Liu et al., 2004). LDL-cholesterol is decreased as well in young male GHR-/- when compared to WT mice (Egecioglu et al., 2006). TG levels are normal to low at young age (Bartke et al., 2004; Egecioglu et al., 2006; Liu et al., 2004; Masternak et al., 2005a) and ApoB levels are also low (Egecioglu et al., 2006). Regarding FFA, normal levels were reported for old GHR-/- mice (Masternak et al., 2005a). Fat accumulation in the liver is also altered in GHR-/- mice. Compared to WT, GHR-/- mice show lower free and higher esterified cholesterol, together with higher TG content in the liver (Bartke et al., 2004; Berryman et al., 2010). When fat accumulation was measured in skeletal muscle, however, no change from WT was observed in TG or fatty acid contents in older male GHR-/- mice (Masternak et al., 2005a). Overall, low levels of cholesterol, TG and FFA are signs of a healthy lipid
profile, probably contributing to the extended lifespan of GHR/-/- mice. How increased fat accumulation in the liver fits into this scheme remains to be understood.

**Thyroid hormones**

Thyroid hormone levels reported for GHR/-/- mice show gender differences. Levels of T₃ and T₄ are lower in four to five-month-old female GHR/-/- mice than controls, but the T₃/T₄ ratio is not different from WT mice, suggesting hypothyroidism (Hauck et al., 2001). In contrast, five to 12-month-old male GHR/-/- mice have elevated total T₃ and normal T₄, resulting in an increased T₃/T₄ ratio (Westbrook et al., 2009). On one hand, the hypothyroidism observed in females is consistent with enhanced fat accumulation in GHR/-/- mice. On the other hand, high thyroid hormone levels, as seen in GHR/-/- males, are consistent with their increased metabolic rate (see below). This paradox reflects the contradicting phenotype of obesity with enhanced insulin sensitivity and longevity. More studies are needed to confirm these findings and determine whether the differences found are dependent on the gender or the age of the animals.

**Other biochemical profiles**

A variety of other metabolic parameters have been measured in GHR/-/- mice. Circulating levels of calcium, bilirubin, uric acid, creatinine, gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP) and total protein are normal, whereas chloride, creatine kinase (CK), alanine aminotransferase (ALT/GPT), urea and albumin are increased in GHR/-/- as compared to WT mice (Liu et al., 2004). Increased levels of CK and ALT might be due to enhanced lipid accumulation in the liver, although normal GGT and ALP suggest liver function is not significantly affected. In addition, potassium levels
are normal or increased and sodium levels are normal or decreased (Egecioglu et al., 2007; Liu et al., 2004). Decreased plasma renin and normal aldosterone levels have also been reported for GHR-/- mice (Egecioglu et al., 2007). Although renin levels are low, normal aldosterone levels are consistent with normal blood pressure and overall cardiac health (Egecioglu et al., 2007).

In summary, compared to WT, GHR-/- mice display constantly low insulin levels, whereas glucose and glucagon values start low and normalize at older age. Corticosteroids are high (at least in males), as are leptin and adiponectin. Lipid profiles generally show decreased cholesterol (total, LDL and HDL) and TG, and normal FFA. Gender differences exist in thyroid hormone levels, with increased levels in males and decreased in females. Also, GHR-/- mice show mostly normal levels of ions and liver enzymes, with some exceptions (chloride, CK, ALT, etc.).

Metabolic changes in the GHR-/- mouse

Glucose metabolism

As discussed in the previous sections, GHR-/- mice show low to normal glucose levels and very low circulating insulin throughout their lifespan. The increased insulin sensitivity observed in GHR-/- mice is likely due to the removal of GH’s anti-insulin activity in these mice.

GHR-/- mice exhibit specific alterations in pancreas structure. Compared to controls, GHR-/- mice display smaller islet and islet cell size (even after normalizing for body weight) (Guo et al., 2005; Liu et al., 2004). Within cell types, β-cell mass is
decreased in GHR-/- mice, together with insulin content and insulin mRNA. These results are consistent with lower circulating insulin levels and suggest lower insulin production capabilities in GHR-/- mice compared to WT. In fact, lower insulin secretion, as compared to controls, is observed in GHR-/- mice after a glucose load (Guo et al., 2005), suggesting glucose intolerance in these animals. In this regard, it is worth noting that GHR-/- mice are not normally exposed to such high amounts of circulating glucose, and therefore their lower insulin secretion is usually sufficient for proper glucose clearance in physiological conditions.

*Insulin signaling*

The enhanced obesity of GHR-/- mice is consistent with a lack of GH lipolytic action, as already explained. The coexistence of obesity and improved insulin sensitivity is a hallmark of GHR-/- mice and raises interesting questions about the regulation of metabolism in these animals.

As stated above, increased insulin sensitivity is consistent with the lack of GH’s anti-insulin activity in GHR-/- mice. However, the mechanisms by which GH antagonizes insulin’s actions are not entirely clear. One hypothesis involves the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), named p85. This protein has several isoforms (p85α, p85β, p55α, p50α) that interact with the catalytic subunit of PI3K, named p110. Interestingly, only the p85α regulatory subunit of PI3K has been linked to insulin resistance when expressed in excess of p110; in mice, enhanced GH action has been associated with an upregulation of p85α in WAT and skeletal muscle (Barbour et al., 2005; del Rincon et al., 2007). This results in a high proportion of free
(inactive) p85α monomers, which can blunt insulin-induced signal transduction by competing with (active) p85-p110 heterodimers for association to tyrosine-phosphorylated IRS-1 (pY-IRS-1). Low p85α levels in mice result in increased insulin sensitivity, even when p85β levels are normal (Barbour et al., 2005; del Rincon et al., 2007). Unfortunately, p85α levels have not been measured in GHR-/- mice. Nevertheless, in humans, a short-term GH infusion had no effect on the insulin-stimulated increase in IRS-1-associated PI3K activity in skeletal muscle (Jessen et al., 2005), demonstrating that more information is necessary to unveil the different mechanisms by which GH might exert its insulin antagonistic actions.

In an effort to explain the molecular mechanisms behind the increased insulin sensitivity observed in GHR-/- mice, the levels and activation of the IR and downstream molecules in the insulin signaling cascade have been characterized in GHR-/- liver, skeletal muscle and heart, displaying interesting tissue and age-specific variations (reviewed in List et al., 2010; and in Sackmann-Sala et al., in press). Unfortunately, WAT has not been included in those studies. In summary, the apparent degree of activation of the insulin signaling cascade in different organs of GHR-/- mice suggests that the high insulin sensitivity in these mice is not reflected uniformly across all insulin-responsive organs. In fact, molecules in the insulin signaling cascade show variable levels of activation in the liver, heart and skeletal muscle of GHR-/- mice. Interestingly, at young ages the liver shows normal activation of the signaling cascade (Dominici et al., 2000), but data from older animals suggest decreased insulin signaling in GHR-/- mice, beyond WT levels (Al-Regaiey et al., 2005). In contrast, heart and skeletal muscle of GHR-/-
mice display enhanced insulin responsiveness as suggested by increased levels of activation of signaling molecules in young adult and older mice (Al-Regaiey et al., 2007; Bonkowski et al., 2009; Giani et al., 2008). Given that insulin acts through numerous and varied mechanisms, further research is necessary to thoroughly characterize the insulin sensitivity in each of these organs. Also, the characterization of insulin responsiveness in different adipose depots of GHR-/- mice would certainly add to the understanding of the unique physiology of these mice.

**Effects of diets on glucose homeostasis and lipid profiles**

GHR-/- mice have been fed various diets, mostly to study effects on longevity and glucose homeostasis. CR has been the most commonly utilized dietary manipulation; several reports have analyzed the effects of CR on longevity, insulin sensitivity, components of the insulin signaling cascade and PPAR and RXR isoforms in liver, skeletal muscle and heart (Al-Regaiey et al., 2007; Bonkowski et al., 2009, Arum, 2009 #4145; Giani et al., 2008; Masternak et al., 2005a; Masternak et al., 2005b; Masternak et al., 2005c; Masternak et al., 2006). In contrast to several other animal models, CR does not extend longevity in GHR-/- mice (Bonkowski et al., 2006b).

The effects of HF feeding on body fat of GHR-/- mice have also been evaluated. Interestingly, GHR-/- mice are not resistant to weight gain induced by a high fat diet, but their glucose and insulin levels remain significantly lower than controls even after the weight gain (Berryman et al., 2006; Robertson et al., 2006). On the other hand, soy-derived diets affect lipid and glucose levels differently, with a genotype-specific increase
in cholesterol and glucose tolerance only with a high (as opposed to a low) isoflavone diet (Bartke et al., 2004).

Proteomics

Techniques used to study the entire pool of proteins expressed in a certain tissue or cell type are collectively referred to as proteomics. The classical proteomic method involves two-dimensional gel electrophoresis (2DE) followed by mass spectrometry (MS) (Kopchick et al., 2007). 2DE consists of a first step of isoelectric focusing (IEF), where the proteins in solution are separated depending on their isoelectric point (pI) and a second step of polyacrylamide gel electrophoresis with protein denaturation by sodium dodecyl sulfate (SDS-PAGE). This second step (second dimension) is performed such that the migration of the proteins (with migration rates depending on each protein’s molecular weight) is orthogonal to the direction of migration of the IEF (first dimension).

Advantages and disadvantages of 2DE

In a recent review of the application of proteomics to the study of diabetes, Scott et al. (2005) commented on the pros and cons of 2DE, summarized here:

Advantages

- Best validated technique available to study whole proteins
- Available stains allow for quantitative measurements
- Splice variants and post-translational modifications can be identified
Disadvantages

- Inadequate resolution of membrane proteins and those very acidic, basic or hydrophobic.
- Low abundance proteins may be below detection limits (nanogram range)

Proteomics of AT

Proteomic approaches have only recently been applied to the study of AT. Despite 2DE being the most commonly used proteomic method, researchers have also studied AT using other proteomic techniques, such as 1DE (SDS-PAGE) (Alvarez-Llamas et al., 2007; Bluher et al., 2004; Hansson et al., 2006; Hausman et al., 2006; Kratchmarova et al., 2002; Subramanian et al., 2004), or liquid chromatography (LC) (Aoki et al., 2007; X. Chen et al., 2005), coupled to MS. However, unlike these techniques, 2DE provides information about both pI and MW of the resolved proteins, allowing for differentiation between protein isoforms. Also, nonequilibrium pH gradient electrophoresis (NEPHGE) has been used by others (Celis et al., 2005; Lanne et al., 2006) as the first separation step, to resolve proteins with very basic pI (7.5 – 11.0), which are not well resolved by IEF. Finally, peptide sequencing rather than identification by MS has also been applied after 2DE of WAT samples (Choi et al., 2004; Xu et al., 2002). The types of AT samples utilized in these studies are summarized in Table 5. Regarding depot-specific differences, only a few proteomic studies have been reported to date. Omental and SC WAT have been compared in obese individuals, displaying differences in various proteins involved in glucose and lipid metabolism, protein synthesis, stress response, etc. (Perez-Perez et
al., 2009). In addition, epicardial AT has been compared to SC fat in humans, suggesting differences in oxidative stress between the two depots (Salgado-Somoza et al., 2010). Recently, Peinado et al. (2010) compared isolated adipocytes and stromovascular fractions from abdominal SC and VISC fat of lean individuals, reporting no differences between adipocytes of each location. In contrast, the stromovascular fraction revealed differences in the levels of stress resistant, cytoskeletal and redox proteins, suggesting that the major differences between depots are originated in this fraction (Peinado et al., 2010).
Table 5

*Reports of WAT Studies Using Proteomics*

<table>
<thead>
<tr>
<th>Sample</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3-L1 cells</td>
<td></td>
</tr>
<tr>
<td>- Cell lysates or culture media</td>
<td>(Alvarez-Llamas et al., 2007; Choi et al., 2004; Kratchmarova et al., 2002; Renes et al., 2005; Wang et al., 2004; Welsh et al., 2004; Wilson-Fritch et al., 2003; Xu et al., 2002; Zvonic et al., 2007)</td>
</tr>
<tr>
<td>- Secretory microvesicles</td>
<td>(Aoki et al., 2007)</td>
</tr>
<tr>
<td>- Lipid droplets</td>
<td>(Subramanian et al., 2004)</td>
</tr>
<tr>
<td>WAT samples from</td>
<td></td>
</tr>
<tr>
<td>Rodents (mainly gonadal WAT)</td>
<td></td>
</tr>
<tr>
<td>- WT C57Bl6 mice</td>
<td>(Sanchez et al., 2001; Schmid et al., 2004)</td>
</tr>
<tr>
<td>- Ob/ob (leptin deficient) mice</td>
<td>(Lanne et al., 2006; Lanne et al., 2001; Sanchez et al., 2003)</td>
</tr>
<tr>
<td>- PPARα-null mice</td>
<td>(Tong et al., 2005)</td>
</tr>
<tr>
<td>- HSL-null mice</td>
<td>(Birner-Gruenberger et al., 2005; Hansson et al., 2006)</td>
</tr>
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Table 5: continued

<table>
<thead>
<tr>
<th>Sample</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIRRKO (fat-specific insulin receptor knockout) mice</td>
<td>(Bluher et al., 2004)</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>(Barcelo-Batllori et al., 2005)</td>
</tr>
<tr>
<td>Obese Zucker rats</td>
<td>(Lanne et al., 2006)</td>
</tr>
<tr>
<td>Charles River CD (Sprague-Dawley-derived) rats</td>
<td>(X. Chen et al., 2005)</td>
</tr>
<tr>
<td>Pig fetuses and neonates</td>
<td>(Hausman et al., 2006)</td>
</tr>
<tr>
<td>Humans</td>
<td></td>
</tr>
<tr>
<td>Abdominal WAT (subcutaneous and/or intra-abdominal)</td>
<td>(Alvarez-Llamas et al., 2007; Corton et al., 2004; DeLany et al., 2005; Peinado et al., 2010; Perez-Perez et al., 2009; Salgado-Somoza et al.; Xiao et al., 2006)</td>
</tr>
<tr>
<td>Epicardial WAT</td>
<td>(Salgado-Somoza et al.)</td>
</tr>
<tr>
<td>Thigh WAT</td>
<td>(Boden et al., 2008)</td>
</tr>
<tr>
<td>Mammary gland WAT</td>
<td>(Celis et al., 2005)</td>
</tr>
</tbody>
</table>
Before the start of this project, WAT samples had been successfully resolved by 2DE in our laboratory by Palmer et al. (unpublished data). The employed protocol was based on the one used by other members of the laboratory to resolve samples of tissues such as liver, kidney, heart, brain, skin and stomach, and had been modified to adjust to WAT properties. Although the main points of the protocol remain the same for all tissues, each one requires particular alterations according to its differential features. In fact, because WAT contains large quantities of lipids, protein isolation has proven to be more complicated for this tissue than for others (Lanne et al., 2001). Therefore, methods for WAT sample preparation preceding 2DE have been optimized in the last decade.

**Adipose tissue sample preparation**

As stated above, WAT is composed of large amounts of TG, which can make the protein isolation step harder than usual. One of the improvements introduced to WAT proteomics was the use of thiourea in the rehydration solution for IEF, which was shown to increase resolution (Lanne et al., 2001).

Methods for protein extraction from WAT vary. The most commonly used one includes homogenization in lysis buffer followed by centrifugation and collection of the water soluble infranatant (below the floating lipid layer) (Barcelo-Batllori et al., 2005; Birner-Gruenberger et al., 2005; Lanne et al., 2006; Lanne et al., 2001; Tong et al., 2005; Xiao et al., 2006). Other protocols include a step of sonication (Hansson et al., 2006) or gentle shaking (Corton et al., 2004) to improve cell lysis before centrifugation. Snap-
freezing of the samples in liquid nitrogen followed by 48-hr long lyophilization and crushing in a mortar with liquid nitrogen has also been reported in several publications (Sanchez et al., 2001; Sanchez et al., 2003; Schmid et al., 2004). Similarly, Casteilla et al. (2008) suggest simply freezing the sample in liquid nitrogen and reducing to powder to facilitate homogenization.

Most recently, the use of pressure cycling technology was reported to result in higher yields for protein extraction from WAT than pulverization (Smejkal et al., 2007). This new technology consists of rapid cycles of alternatively applied high and low hydrostatic pressures, to promote efficient cell lysis in the sample (Smejkal et al., 2007). Advantages of this method include avoiding the temperature cycles generated by sonication, which can compromise protein solubility, and possibly improving solubilization of hydrophobic proteins (Smejkal et al., 2007).

Proteomic studies of WAT carried out in our laboratory (Palmer et al., unpublished data) have included sample preparation by homogenization in lysis buffer, followed by sonication, centrifugation and collection of the aqueous phase. For the present work, the three described methods for sample preparation (mechanical homogenization in lysis buffer plus sonication, pressure cycling and lyophilization) were tested and compared, with the first two yielding the best results (see Chapter 3).
CHAPTER 2: RESEARCH OBJECTIVES

Goals and hypotheses

The goal of this project was to establish the differences in protein profiles present in WAT of distinct adipose depots. One SC and three intra-abdominal adipose depots were studied in different WT and GHR-/- mice (see below). As discussed in Chapter 1, intra-abdominal adipose depots are generally more closely associated with the metabolic syndrome than SC depots. Therefore, a thorough characterization of the protein expression profiles of these depots might serve to explain the molecular mechanisms behind the development of insulin resistance, type 2 diabetes and cardiovascular disease generally observed in obese individuals. Physiologically relevant differences not associated to obesity could also be revealed by comparing protein profiles of different WAT depots. To this end, the proteomes of the four different adipose depots (SC (inguinal) and intra-abdominal (retroperitoneal, mesenteric and epididymal)) were first compared in adult (12 months old) male WT C57Bl/6J mice. The information obtained from this comparison is described in Chapter 4. In this case, the hypothesis tested (Hypothesis 1) was that distinct adipose depots display differences in their protein expression profiles. The second stage tested the hypothesis that proteomes of different adipose depots change with age in a depot-specific manner (Hypothesis 2). In other words, protein expression profiles of certain adipose depots are affected by age in a way that is different to the protein expression profile changes observed with age in other depots. To test this hypothesis, the proteomes of identical adipose depots were compared between WT mice of different ages (12 months vs. 24 months old). The results for this
part of the project are described in Chapter 5. Finally, the WAT proteomes of 12 and 24-month-old GHR-/- mice were analyzed and compared to those of WT mice. Thus, the last part of the project dealt with genotype-specific differences present in the protein expression profiles of the four adipose depots, in addition to age- and depot-specific differences. As described in Chapter 1, GHR-/- mice have a distinctive distribution of AT, predominantly accumulated in the SC region; however, they present a favorable metabolic phenotype in spite of obesity. In this part of the project the hypothesis tested (Hypothesis 3) stated that, compared to WT, GHR-/- mice display different WAT proteomes in individual adipose depots that depend on the age of the animal and the specific adipose depot under consideration. The description of the results of this analysis is included in Chapter 6.

As mentioned previously, reports of 2DE of WAT in the literature include different homogenization procedures for protein isolation from WAT samples. Also, there are varied conditions used for the second dimension (SDS-PAGE) step. Therefore, in a preliminary phase of this project, the sample preparation and 2DE technique was optimized to obtain the best resolution possible in the protein gels. These experiments are described in Chapter 3.

Fat depots studied

The WAT depots that are generally studied in mice include inguinal, gonadal, retroperitoneal, and more recently, mesenteric. This study included samples of these four WAT depots obtained from male mice. As a group, these fat depots represent all WAT
types: SC (inguinal), intra-abdominal VISC (mesenteric) and intra-abdominal non-VISC (retroperitoneal and epididymal). Although mouse AT samples are commonly used as a model for AT in humans, there are several differences in murine and human AT, as summarized by Casteilla et al. (2008) and shown in Table 6. Mouse models are, despite these major differences, a great tool for the study of AT because of the feasibility to create transgenic and gene deficient mice. When interpreting data obtained from mouse animal models, however, these differences must always be taken into consideration when extrapolating results to human physiology/pathology.
Table 6

*Main Features Differentiating ATs in Rats, Mice and Humans*

<table>
<thead>
<tr>
<th></th>
<th>Rats and mice</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of fat pad:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interscapular</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Periovarian</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Epididymal</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Persistence of brown fat in adults</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>Convertible features</td>
<td>Mice &gt; rats</td>
<td></td>
</tr>
<tr>
<td>BAT → WAT</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>WAT → BAT</td>
<td>+++</td>
<td>?</td>
</tr>
</tbody>
</table>

Ing > PO > RP > Ep

<table>
<thead>
<tr>
<th>Main site of lipogenesis</th>
<th>AT</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose transport sensitive to insulin</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Catecholamine-stimulated lipolysis</td>
<td>$\beta_1$, $\beta_2$, $\beta_3$ (+)</td>
<td>$\beta_1$, $\beta_2$ (+)</td>
</tr>
</tbody>
</table>

Ing, inguinal; PO, periovarian; RP, retroperitoneal; Ep, epididymal. Reproduced with kind permission from Springer Science+Business Media: *Adipose tissue protocols*, Choosing an adipose tissue depot for sampling: Factors in selection and depot specificity, 2nd ed., vol. 456, 2008, pp. 29, Casteilla, L., Penicaud, L., Cousin, B., & Calise, D, Table 2.5. Note: $\beta_3$-adrenoceptors are also present in humans, although they play a minor role in catecholamine-stimulated lipolysis (Arner, 2005).

As shown in Table 6, one of the WAT depots included in this study (epididymal) is not present in humans. The role of this WAT depot in mice, however, has been suggested to be analogous to that of the human omental depot (Thomou et al., 2010). In
fact, in bipeds, the omentum hangs down from the stomach and serves a protective role on internal organs (Thomou et al., 2010). This role is analogous to that of epididymal (or parametrial) fat in quadrupeds (Thomou et al., 2010), which coats most of the ventral surface of the peritoneal cavity. These patching or bandage-like functions are linked to an enhanced immune function in these WAT depots, with high production of inflammatory cytokines (Wozniak et al., 2009). In this respect, given the association of VISC WAT inflammation and the metabolic syndrome (Wisse, 2004), epididymal WAT could be considered a VISC-like depot. However, as noted before, epididymal WAT is not a VISC depot, given that it does not drain into the portal vein. In fact, marked differences exist between human omental and mouse epididymal WAT regarding circulation. While the omental depot drains into the portal vein, the epididymal depot drains to peripheral vessels (Cinti, 2001). The differences in circulation might lead to distinct metabolic effects of these two depots, given that upon activation of lipolysis, only omental fatty acids will rapidly reach the liver. Therefore, the VISC-like characteristics of the epididymal WAT depot might only be associated to its increased immune function.

One further difference between human omental and mouse epididymal WAT relates to adipocyte sizes. In humans, omental WAT normally displays smaller adipocytes than SC WAT (although in obesity, omental adipocytes are enlarged and reach sizes similar to those of SC cells) (Zhang et al., 2002). In contrast, adipocytes in WT mouse epididymal WAT are larger than in SC depots (Kelder et al., 2007; Zhang et al., 2002). Given the influence of adipocyte size on the secretion of leptin, adiponectin and other inflammatory cytokines (Ando et al., 2005), the distinctions between cell sizes
in WAT depots of humans and mice might have critical implications on their respective secretory profiles. For example, leptin mRNA in humans is higher in SC than omental fat (Montague et al., 1997), whereas in mice leptin mRNA levels are lower in SC than epididymal and retroperitoneal depots, mainly due to varying cell volumes (Zhang et al., 2002). Therefore, mouse epididymal WAT is only partly comparable to human omental WAT, with similarities in protective/immune function and differences in blood supply and relative cell size.

Finally, to avoid added variability in the WAT depots studied, only male mice were analyzed. Gender differences, as discussed above, have a large impact on AT physiology. A review of gender differences in fat metabolism in humans has been reported elsewhere (Blaak, 2001). In addition, dissections were carried out for all groups in the morning, between 8 am and 12 am, to avoid variability due to circadian rhythms (Ando et al., 2005) (see Chapter 1).
CHAPTER 3: OPTIMIZATION OF SAMPLE PREPARATION PROCEDURES AND SDS-PAGE CONDITIONS FOR 2DE OF WAT

Abstract

The high content of lipids present in WAT represents a caveat for protein isolation and resolution by 2DE. Various reports in the literature utilize differing methods of WAT sample homogenization, delipidation and 2DE formats. Therefore, we set out to compare the available methods to reveal the one that provides the best resolution in a proteomic analysis of WAT. Protein isolation techniques tested included mechanical homogenization plus sonication, pressure cycling, and lyophilization plus crushing in mortar with liquid nitrogen. An extra delipidation step involving extraction with acetone/ether was also evaluated. Finally, four SDS-PAGE formats were compared, including combinations of fixed (12.5 or 15%) or gradient (10-20%) polyacrylamide concentrations and large or small gel sizes (which accommodate broad or narrow pI ranges respectively). Our results showed that the best resolution of WAT proteins was obtained both via homogenization plus sonication and via pressure cycling, with no improvement provided by the delipidation step. In addition, small gels (pI 5-8) with constant polyacrylamide concentration (15%) yielded the best resolution of WAT proteins among the SDS-PAGE formats tested. These conditions should be utilized in future proteomic analyses of WAT by 2DE. Our results provide valuable information for those intending to use 2DE for the study of WAT proteins.
Introduction

Proteins from WAT are the target of many studies to understand, among other things, how this tissue regulates metabolism. However, the high content of lipids in WAT renders protein isolation a difficult task. In this study, several methods of protein extraction, sample preparation and two-dimensional gel electrophoresis conditions were compared.

Different reports in the literature include one of three procedures for homogenization of WAT and protein extraction: a) homogenization in lysis buffer, centrifugation and collection of aqueous phase (Barcelo-Batllo et al., 2005; Birner-Gruenberger et al., 2005; Lanne et al., 2006; Lanne et al., 2001; Tong et al., 2005; Xiao et al., 2006) (may also include sonication (Hansson et al., 2006) or gentle shaking (Corton et al., 2004)); b) freezing, 48 hr-lyophilization and crushing in mortar with liquid N$_2$ (Sanchez et al., 2001; Sanchez et al., 2003; Schmid et al., 2004); and c) pressure cycling – high and low hydrostatic pressures applied alternatively in rapid cycles (Smejkal et al., 2007).

During the last 10 years, mechanical homogenization has been the standard method of use in our laboratory for protein isolation prior to proteomic analyses of a variety of tissues (liver, kidney, brain, WAT, etc.) (Kopchick et al., 2002; List et al., 2007a; List et al., 2007b; and Cruz-Topete, Sackmann-Sala, Palmer (unpublished results)). For this reason, this technique was chosen as a basis for comparison with either lyophilization or pressure cycling technology for protein isolation from WAT. In addition, the improvement in gel resolution produced by delipidation of the samples (via
ether/acetone extraction) was evaluated as a complementary step that could be added to the protein isolation protocol.

Regarding the second dimension of the 2DE technique, different authors use conditions that involve constant or gradient polyacrylamide gels of varying sizes. Larger gel sizes can accommodate broad pH ranges (3-10), and can resolve proteins within a wider range of molecular weight, especially using gradient gels. In contrast, smaller gels provide resolution of narrower ranges but are easier to handle and faster to run, characteristics that can be valuable if the decrease in the number of proteins resolved is not significant. Because each condition presents distinct advantages and disadvantages, we evaluated the resolution of WAT proteins using four different SDS-PAGE formats: large (20 cm) and small (8 cm) gels with constant (12.5 or 15%) or gradient (10-20%) polyacrylamide concentrations.

Our results show that mechanical homogenization plus sonication is as good as pressure cycling for protein isolation from WAT samples. Interestingly, extraction of lipids using acetone/ether did not improve resolution of proteins isolated by mechanical homogenization plus sonication. In addition, small gels of constant polyacrylamide concentration provided the best resolution of WAT proteins by 2DE. The information obtained in this study is significantly useful for future proteomic studies of WAT.
Materials and Methods

Samples

Four WAT depots (inguinal, retroperitoneal, mesenteric, and epididymal) samples of three healthy male WT C57BL/6J mice were obtained. Mice were kept on a 12-hour light/dark cycle with food and water provided ad libitum. The day of dissection, mice were sacrificed by cervical dislocation. Procedures were approved by the Ohio University Animal Care and Utilization Committee. In all cases, samples were dissected, immediately frozen in liquid nitrogen, and stored at -80 °C. Before processing, each sample was thawed and divided in two similar portions (one for each of two treatments).

Homogenization in lysis buffer, sonication, centrifugation and collection of aqueous phase.

This procedure was standard for proteomic analysis of most tissues (liver, kidney, etc.) in the laboratory. Samples were homogenized in three to four volumes of lysis buffer (sample buffer) containing 7 M urea, 2 M thiourea, 1% w/v sulfobetaine-10, 3% w/v CHAPS, 0.25% v/v Bio-Lyte 3/10 ampholytes (Bio-Rad Laboratories Inc., Hercules, CA), and 1.5% v/v protease inhibitor cocktail (Sigma, St. Lewis, MO, cat.# P8340).

After mechanic homogenization using a Polytron PTA 20 TM homogenizer (Kinematica, Inc., Bohemia, NY), samples were sonicated briefly (~10 seconds) using a Model 100 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and incubated at ~30-35 °C for 15 min prior to centrifugation. Next, samples were centrifuged at 16,000 g for 45 min (14,000 rpm on a bench-top centrifuge), which resulted in the separation of three
phases: a floating layer of lipid on the top, an aqueous solution that contained the isolated proteins in the middle, and a small pellet of large cell debris on the bottom. Fatty layers were separated with a spatula and stored at -80 °C; aqueous infratants containing the isolated proteins were transferred to clean tubes; pellets were discarded.

Freezing, 48 hr-lyophilization and crushing in mortar with liquid N₂

Lyophilization was performed in a Hetovac VR-1 (Heto-Holten A/S, Allerod, Denmark). Samples were snap-frozen in liquid N₂ before the lyophilization step, which was carried out at room temperature and negative pressure for 48 hours. After lyophilization, the samples were crushed in a mortar with liquid N₂ and a thick paste was obtained. The paste was resuspended using ddH₂O up to the initial sample weight. Given the high content of oil remaining in the lyophilized samples, a centrifugation step was performed for 30 min at 16,000 g and room temperature. The fatty layers were separated with a spatula and the infratants containing the isolated proteins were transferred to clean tubes for further processing.

Pressure cycling: high and low hydrostatic pressures applied alternatively in rapid cycles.

A Barocycler NEP2320 kindly provided by Pressure Biosciences, Inc. (West Bridgewater, MA) was used for this experiment. The protocol used consisted of 20 cycles, where a pressure of 35kpsi (~241,000 kPa) was applied for 20 sec followed by 20
sec at ambient pressure (~100 kPa). A ProteoSolve kit for lipid extraction was utilized as recommended by Pressure Biosciences, Inc. Treated samples were resuspended in sample buffer.

**Delipidation**

The effects of a delipidation step on gel resolution were evaluated for the protein samples. The delipidation step (acetone/ether extraction) was performed after sonication and incubation at ~30-35 °C (before centrifugation) during the sample preparation process. The delipidation protocol involved three extraction steps:

a) Addition of >10 ml of cold acetone (-80 °C) per milliliter of sample, overnight incubation at -20 °C, centrifugation at 4 °C for 25 min and discarding of supernatant

b) Resuspension of pellet via extensive vortexing in >10 ml of 1:1 acetone:ether per milliliter of original sample, centrifugation at 4 °C for 25 min and discarding of supernatant

c) Resuspension of pellet via extensive vortexing in >10 ml of ether per milliliter of original sample, centrifugation at 4 °C for 25 min and discarding of supernatant. Drying of pellet under hood and resuspension in sample buffer up to original volume of sample.

The success of the delipidation process was confirmed by the absence of a fatty layer after the centrifugation (16,000 g for 30 min at room temperature) of the samples.
First dimension

Protein concentration was measured in all samples using the Bio-Rad Protein Assay. For the first dimension, 150 µg of protein were diluted in 350 µl of sample buffer. Tributylphosphine was used to reduce disulfide bonds and iodoacetamide was added to alkylate reduced cysteine residues. Reduced and alkylated samples were loaded onto immobilized pH gradient (IPG) strips (17 cm, pH 3-10 linear, Bio-Rad) and rehydrated passively for 2 h at room temperature. IEF was run in a PROTEAN IEF cell (Bio-Rad), with 12 h of rehydration at 50 V and separation by slow voltage increase to 10,000 V for 3 h followed by rapid voltage increase up to 60,000 V·h.

Second dimension

After IEF, IPG strips were incubated for 45 min in equilibration buffer (0.375 M Tris-HCl pH 8.8, 6 M urea, 2% w/v SDS, 20% v/v glycerol and bromophenol blue). During equilibration, the buffer was replaced with fresh buffer at 10 and 25 min. For the second dimension, different SDS-PAGE formats were compared:

a) 8 cm 15% polyacrylamide gels (pH 5-8)

b) 8 cm 10-20% gradient polyacrylamide gels (pH 5-8)

c) 20 cm 12.5% polyacrylamide gels (pH 3-10)

d) 20 cm 10-20% gradient polyacrylamide gels (pH 3-10)

For small (8 cm) gels, 4.5 cm were cut from each end of the 17-cm long IPG strips, to obtain the middle 8-cm segment (pH 5-8). For the large (20 cm) gels, the strips were left intact. Gels with constant concentration of polyacrylamide were prepared
manually and included 4-7% stacking gels; pre-cast 10-20% gradient gels were obtained from Bio-Rad. SDS-PAGE of small gels was run in a Mini-PROTEAN 3 cell (Bio-Rad) at 25 mA/gel for 270 V·h. Large gels were run in a PROTEAN II XL cell (Bio-Rad) at 75 mA/gel for ~3 h. After fixing and washing, gels were stained with SYPRO Orange (Molecular Probes, Inc., Eugene, OR) and scanned using a PharosFX Plus Molecular Imager (Bio-Rad). The excitation wavelength used was 488 nm and emission was detected at 605 nm.

Results

Comparison of protein isolation methods

Pressure cycling and mechanical homogenization plus sonication achieved similar resolution, with a couple of slight differences (Figure 13). In contrast, the lyophilization process failed to isolate proteins belonging to the main two ‘trains’ (protein spots of similar molecular weight but varying pI that form a horizontal dotted line) in the gel (Figure 13). In addition, there were no differences between samples subjected to lipid extraction with acetone/ether and those that were directly centrifuged after sonication (regular sample preparation) (Figure 14).
Figure 13. Comparison of protein isolation methods. Green outlines highlight differences in spot intensities observed when mechanical homogenization plus sonication was compared to pressure cycling. Red outlines mark differences in spot intensities observed when mechanical homogenization plus sonication was compared to lyophilization. Abbreviations: MW, molecular weight, pl, isoelectric point.
Figure 14. Comparison of gel images from samples subjected or not to delipidation using ether/acetone. Abbreviations: MW, molecular weight; pI, isoelectric point.
SDS-PAGE conditions

The small 15% gels showed the best resolution of the four conditions tested (Figure 15). Spots resolved in small 10-20% gradient gels were too compacted, impairing image analysis (Figure 15A). Large gels displayed lower resolving power overall, with blurry spots and increased vertical streaking. Moreover, only a few proteins seemed to be present in the acidic (pI 3-5) and basic (pI 8-10) ends of these gels (Figure 15B).
Figure 15. Resolution of WAT proteins from epididymal (left) and inguinal (right) depots by 2DE using different SDS-PAGE formats. A. Small gels (8 cm) with constant (15%) polyacrylamide concentration (top) or a concentration gradient (10-20%, bottom). B. Large gels (20 cm) with constant (12.5%) polyacrylamide concentration (top) or a concentration gradient (10-20%, bottom). Abbreviations: MW, molecular weight; pI, isoelectric point.
Discussion

WAT presents a challenge for isolation of proteins because of the high amount of lipid contained in this tissue. There are various ways of treating WAT samples for 2DE reported in the literature (Barcelo-Batllori et al., 2005; Birner-Gruenberger et al., 2005; Corton et al., 2004; Hansson et al., 2006; Lanne et al., 2006; Lanne et al., 2001; Sanchez et al., 2001; Sanchez et al., 2003; Schmid et al., 2004; Smejkal et al., 2007; Tong et al., 2005; Xiao et al., 2006). Mechanic homogenization plus sonication is the method of choice for use in our laboratory for a myriad of tissues, including WAT (Kopchick et al., 2002; List et al., 2007a; List et al., 2007b; and Cruz-Topete, Sackmann-Sala, Palmer (unpublished results)). To test whether other homogenization methods yielded higher resolution in 2DE gels, pressure cycling and lyophilization were compared to mechanical homogenization plus sonication. The best resolution was obtained when pressure cycling or mechanical homogenization plus sonication were used as homogenizing methods (Figure 13). The differences in resolution obtained using these two techniques were very few and only slightly noticeable. On the other hand, when lyophilization was used, some of the main spot “trains” in the gels were very faint. Apparently, lyophilization fails to isolate the proteins in these trains, demonstrating lower resolving power than the other two homogenizing methods. The instrumentation necessary for mechanical homogenization plus sonication was readily available in the laboratory, and our experience with this homogenization method was ample. In contrast we did not own a barocycler, which is necessary for pressure cycling. Therefore, the method of choice in
following experiments involving WAT was our standard mechanical homogenization plus sonication.

The next variation tested for the processing of WAT samples was delipidation by extraction with acetone/ether. Surprisingly, there was no improvement in resolution upon delipidation of samples, suggesting that the centrifugation step performed after homogenization and sonication of the samples is enough to extract the lipids from the protein solution, providing good resolution in the protein gels. Importantly, omitting the extraction procedures saves almost one day in the sample treatment process.

Regarding SDS-PAGE conditions, the best 2D-gel images were obtained using small (8 cm) 15% polyacrylamide gels. As observed in large gels, with a range of pI = 3-10, the resolution beyond the pI range 5-8 is poor. No proteins are visible outside the 5-8 pI range. Therefore, large gels do not provide additional information than that seen in small gels. In addition, no improvement in resolution is observed when using 10-20% gradient gels as opposed to a constant polyacrylamide concentration (15%). We expected to be able to improve resolution of proteins in the high molecular weight range (>60 kDa) by using gradient gels; many metabolic enzymes (HSL (83 kDa), phosphoenolpyruvate carboxykinase (69kDa), etc.), hormone receptors (GHR (73 kDa), IR (156 kDa), IGF-1 receptor (156kDa), etc.), and signaling molecules (e.g. p85 subunit of PI3K, (83kDa)) are within that molecular weight range (although receptors are hard to isolate given their tight relation to the cell membrane). However, even when using gradient gels, the resolution of the high molecular weight region in the gel was poor. Therefore, the small 15% polyacrylamide gels were used in following experiments.
The SWISS-2DPAGE database (Hoogland et al., 2004) provides a map of murine WAT proteins resolved in a 2D-gel (Figure 16). The WAT 2D-gel image featured is the result of the work of Sanchez et al. (2001). The procedures for protein isolation and 2DE used by that group to obtain such gel include lyophilization and large gradient polyacrylamide gels. These conditions were tested in our study and, in our hands, showed inferior resolution than our standard WAT sample treatment protocol. It is interesting to note that, similar to our observations (Figure 13), the WAT 2D-gel image featured by the SWISS-2DPAGE database (Figure 16) lacks the long spot-train at ~45 kDa (that in our gels of samples treated by mechanical homogenization plus sonication spans a pI range from 5.5 to 6.5 and in Figure 16 is only present between pI 5 and 5.5) and the train at ~25 kDa (which in Figure 16 only shows two spots). However, the overall resolution in the gel featured in the SWISS-2DPAGE database is clearly superior to the ones obtained in this study. This difference could be attributed to other differences in the protocol used by Sanchez et al. (2001), such as the reduction and alkylation of disulfide bonds after the IEF step, the staining method utilized (silver stain), the use of IPG strips of non-linear pH range, and/or slight modifications of the sample buffer and IEF conditions, which were not tested in this study. These variations, however, would not be expected to provide the marked improvements in resolution observed in the gel in Figure 16.
In summary, mechanical homogenization plus sonication and pressure cycling were the best procedures among those tested to isolate proteins from WAT samples for 2DE. Centrifugation and separation of the lipids in the fatty layer were enough to delipidate the samples, and there was no improvement in resolution when acetone/ether extraction was included. The best resolution in 2D protein gels was obtained when small (8 cm) gels of constant polyacrylamide concentration (15%) were used. Ironically, this was the original combination of procedures applied to WAT samples before this project was started. It is clear that none of the changes tested need to be applied to the standard protocol utilized.
CHAPTER 4: WHITE ADIPOSE TISSUE IN 12-MONTH-OLD WILD-TYPE MICE: PHENOTYPIC AND PROTEOMIC DIFFERENCES AMONG DEPOTS

Abstract

WAT plays a role in metabolic regulation, immunity, glucocorticoid and steroid hormone synthesis, and other major functions. However, the contributions of individual adipose depots to each of these functions are not always equal. For example, intrinsic differences in endocrine function have been reported for individual WAT depots. To investigate their distinctive characteristics, we profiled proteins in SC (inguinal) and intra-abdominal (mesenteric, epididymal and retroperitoneal) WAT depots of C57BL/6J mice using 2DE followed by MS. WAT depot-specific weight, mean adipocyte size, total protein content and correlations of these variables and spot intensities with plasma insulin, leptin and/or adiponectin levels were also determined. The epididymal depot was the largest in mass, and the mesenteric depot had the highest protein content of the four WAT depots analyzed. Mean adipocyte size was highest in epididymal and lowest in mesenteric and inguinal depots. Proteomic results showed major differences in protein profiles in inguinal as compared to mesenteric and epididymal depots. Inguinal WAT displayed lower expression of enzymes involved in ATP generation, glucose and lipid metabolism, and decreased levels of antioxidant proteins. These results are consistent with lower TG turnover and consequently lower production of ROS in SC as compared to mesenteric and epididymal WAT. Together, these data reveal that each WAT depot possesses distinct phenotypic characteristics and proteomic profiles that may ultimately
reflect its unique physiological role. This information adds to the understanding of WAT physiology and the role of different WAT depots in metabolism and homeostasis.

Introduction

The role of WAT in the body spans a wide range of functions, including metabolic regulation, inflammation and immunity, glucocorticoid and steroid hormone synthesis, hemostasis, blood pressure regulation, and others (Trayhurn, 2005). Although WAT was historically considered a homogeneous organ, fundamental differences among WAT depots have now been reported (Cartwright et al., 2007; Tran et al., 2008; Wajchenberg et al., 2002). Depot-specific differences include their influence on metabolism (Tran et al., 2008), endocrine function (Wajchenberg et al., 2002), and characteristics of preadipocytes (Cartwright et al., 2007). In general, intra-abdominal WAT has a more negative impact on insulin sensitivity than SC WAT and is associated with cardiovascular disease, stroke, hypertension, type 2 diabetes, hyperlipidemia, and an overall increase in morbidity and mortality (Wajchenberg et al., 2002).

As described in Chapter 1, the levels of various proteins have been measured and compared among WAT depots in humans and rodents (Berndt et al., 2005; Fain et al., 2004; X. Li et al., 2004; McTernan et al., 2002; Wajchenberg, 2000; R. Z. Yang et al., 2006). However, the available data are not sufficient to explain why individual WAT depots have distinct physiological effects. To that end, the broad view of protein expression obtained through a proteomic approach might offer valuable information concerning depot-specific biological activities. Previous studies comparing SC and intra-
abdominal WAT have focused on obese individuals (Perez-Perez et al., 2009), or have separately analyzed isolated adipocytes and stromovascular cells (Peinado et al., 2010). However, a proteomic characterization of intact tissue looking at depot differences in normal physiological conditions has not been performed.

In this study, we used proteomics (2DE followed by MS) to study four WAT depots in ‘normal’ 12-month-old WT C57Bl/6J mice. Given their functional differences, we hypothesized that individual WAT depots would display distinct proteomic profiles. Depot weight, total protein content, adipocyte sizes, and the correlation of these values and protein spot intensities with plasma levels of insulin, leptin and adiponectin were also determined.

Three intra-abdominal (mesenteric, epididymal and retroperitoneal) and one SC (inguinal) WAT depots were included in this study. Among the intra-abdominal depots, only mesenteric WAT drains into the portal vein and therefore can be assumed to be in close “communication” with the liver. The retroperitoneal depot, located behind the kidneys is the only intra-abdominal depot that is outside the peritoneal cavity. The epididymal depot, instead, is the most studied WAT depot given its ease of dissection and considerable size.

This thorough characterization of WAT depots in normal conditions provides valuable information about depot-specific differences, not only between intra-abdominal and SC depots, but even within intra-abdominal depots. Our data reveal that each WAT depot possesses a distinct phenotype that may ultimately reflect its unique physiological role. The differences observed in protein expression patterns among WAT depots will
help find depot-specific targets for diagnosis and treatment of obesity-related diseases, such as type 2 diabetes, cardiovascular disease and cancer.

Materials and Methods

Mice

Male WT C57BL/6J mice (12 months old) were used in this study. Mice were kept on a 12-hour light/dark cycle with food and water provided ad libitum. Procedures were approved by the Ohio University Animal Care and Utilization Committee.

Body weight and body composition

Body weight and body composition were measured on 10 mice one week before sacrifice. Body composition was detected using a quantitative NMR apparatus (Minispec, Bruker Optics, Billerica, MA); two measurements were performed and the average recorded.

Plasma insulin, leptin and adiponectin

Two weeks prior to sacrifice, mice (n=10) were fasted for eight hours and blood from the tail vein was collected in heparinized tubes. Plasma was then separated by centrifugation at 7,000 g for 10 min and stored at -80 °C until processing. Insulin levels were measured using an ELISA kit from ALPCO Diagnostics, Salem, NH (80-INSMSU-E01). Leptin levels were quantified using an ELISA kit from R&D Systems, Inc.,
Minneapolis, MN (MOB00). HMW and total adiponectin levels were measured using an ELISA kit from ALPCO Diagnostics (47-ADPMS-E01).

**WAT depot samples**

Dissections were carried out between 8:00 am and 12:00 pm to avoid variability in protein expression due to circadian rhythms (see Chapter 1). Twelve mice were sacrificed by cervical dislocation and inguinal, retroperitoneal, mesenteric and epididymal WAT were collected and weighed. Mice were randomly divided into two groups of six; WAT samples from six mice were collected for proteomic analysis, while the rest were fixed in 10% formalin and processed for histology (see below). Samples for proteomic analysis were snap-frozen in liquid nitrogen and stored at -80°C until processing.

**Proteomic analysis**

These procedures are based on those described previously (Kopchick et al., 2002; List et al., 2007b; Okada et al., 2010; Qiu et al., 2005; Sackmann-Sala et al., 2009) and the results obtained in the optimization tests described in Chapter 3.

a) **Adipose tissue sample preparation:** WAT samples were thawed, reweighed and diluted in sample buffer containing 7 M urea, 2 M thiourea, 1% w/v sulfobetaine-10, 3% w/v CHAPS, 0.25% v/v Bio-Lyte 3/10 ampholytes (Bio-Rad), and 1.5% v/v protease inhibitor cocktail (Sigma). The volume of buffer used for dilution was 3 ml/g for inguinal, epididymal and retroperitoneal depots and 4 ml/g for the mesenteric
depot. Protein solubilization was achieved through mechanical homogenization followed by brief sonication, incubation at 35 °C for 15 min, and centrifugation at 16,000 g for 45 min at room temperature. Floating lipid layers were removed and infranatants containing the isolated proteins were transferred to clean tubes. Protein concentrations were measured using Bio-Rad Protein Assay. Immediately prior to 2DE, aliquots containing 150 µg of protein were further diluted to 350 µl in sample buffer; disulfide bonds were reduced with tributylphosphine and sulphydryls were alkylated with iodoacetamide.

b) 2DE: For the first dimension, 350 µl of each reduced and alkylated protein solution (see “a)” above) was loaded onto IPG strips (17 cm, pH 3-10 linear, Bio-Rad) and passively rehydrated for two hours at room temperature. Then, IPG strips were placed into a PROTEAN IEF cell (Bio-Rad) for IEF consisting of 12 hours of active rehydration at 50 V and separation by slow voltage increase to 10,000 V for 3 h followed by rapid voltage increase up to 60,000 V·h. Next, strips were equilibrated for 45 min in buffer containing 0.375 M Tris-HCl pH 8.8, 6 M urea, 2% w/v SDS, 20% v/v glycerol and bromophenol blue. After cutting 4.5 cm from both sides of each strip, the middle 8-cm segment (pH 5 to 8) was loaded on a 15% polyacrylamide gel with 4% stacking. SDS-PAGE was performed in a Mini-PROTEAN 3 cell (Bio-Rad) at 25 mA/gel for 270 V·h. After fixing and washing, gels were stained using SYPRO Orange (Sigma) and scanned in a PharosFX Plus Molecular Imager (Bio-Rad). Excitation was performed at 488nm, and emission was detected at 605 nm.
c) *Image analysis:* Spot matching was performed using the image analysis software PDQuest Advanced (version 8.0, Bio-Rad), and all matches were examined manually.

Spot intensities were normalized to total image density in each gel.

*Mass spectrometry*

Protein spots displaying significant intensity changes among depots were manually excised from the gels and sent to Protea Biosciences Inc., Morgantown, WV for analysis by MS and tandem-MS (MSMS) using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF-TOF respectively.

a) *In-gel digestion and sample preparation protocol (Performed by Protea Biosciences Inc.):* Gel spots were diced into small pieces. Gel pieces were dehydrated with acetonitrile and then rehydrated with 50 mM ammonium bicarbonate. Proteins in the gel pieces were reduced and alkylated with 250 mM DTT (60 min at 55 °C) and 650 mM iodoacetamide (60 min in the dark at room temperature), respectively. Digestion was performed with 500 ng of trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was performed using 5% formic acid in 50% acetonitrile (dehydration), followed by rehydration with 50 mM 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 10 mM acetic acid, and re-lyophilized to yield a purified, protein digest extract.
b) **MALDI target spotting protocol (Performed by Protea Biosciences Inc.)**: A C18 ProteaTip was washed and then equilibrated using a 0.1% TFA/50% acetonitrile solution and a 0.1% TFA/2% acetonitrile solution, respectively. The remaining reconstituted protein digest solution in the autosampler vial (~65% of sample) was loaded onto the C18 ProteaTip by aspirating and expelling the sample five to 10 times within the sample vial. The bound sample was washed twice with the 0.1% TFA/2% acetonitrile solution by aspirating and expelling 20 µL of the wash solution five to 10 times. The sample was spotted directly onto a MALDI target that was pre-spotted with 0.6 µL MALDI matrix (CHCA) using 1 µL of an elution solution (0.1% TFA/90% acetonitrile).

c) **MALDI mass spectrometer parameters (Performed by Protea Biosciences Inc.)**: Instrument: ABI 4800 MALDI TOF/TOF analyzer; data acquisition and processing program: 4000 Series Explorer software; MS acquisition in reflector mode positive ion mode; mass range: \(m/z = 850 - 4000\); 400 laser shots per spectrum; minimum S/N = 10 for MS acquisition; 15 strongest precursors chosen for MSMS; minimum S/N = 30 for MSMS precursors; MALDI spot interrogated until at least 4 peaks in the MSMS spectra achieved a S/N = 70.

d) **Database correlation analysis search parameters (Performed by Protea Biosciences Inc.)**: Protein identification from MS and MSMS data: Program for data processing: Applied Biosystems GPS Explorer v3.6; search engine: Mascot (Matrix Science); sample type: gel samples; digestion enzyme: trypsin; species: mouse; I.D focus: biological modifications; database: NCBIInr or Swiss-Prot; search engine: type of
search: combined MS and MSMS; Mascot (Matrix Science); mass values:
monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 0.3 - 1 Da;
maximum missed cleavages: 1-2; variable modifications: oxidation (M),
carbamidomethyl (C), propionamide (C); Exclusion mass list: 870.7, 1151.8, 1358.9,
1795.1, 1910, 2211.4, 2225.4, 2283.5, 2283.5, 2807.7, 3019.8, 3337.7.

**Protein identification (Performed at Ohio University)**

Protein identities obtained by Protea Biosciences (see “d)” above) were verified or revised using the MS and MSMS data obtained and the online softwares Mascot (www.matrixscience.com) and MS-Seeker (www.msseeker.org). Search parameters included the following: **MS**: database: NCBInr; taxonomy: *Mus musculus*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none or carbamidomethyl (C); protein mass: none; peptide tolerance: ±0.1 - 1.2 Da; mass values: MH+;
monoisotopic/average: monoisotopic. **MSMS**: database: NCBInr; taxonomy: *Mus musculus*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none or carbamidomethyl (C); quantitation: none; peptide tolerance: ±0.1 - 1.2 Da; MSMS tolerance: ±0.1 - 0.6 Da; peptide charge: 1+; monoisotopic/average: monoisotopic; precursor m/z: none; instrument: MALDI-TOF-TOF. Variable modifications that were included in separate and combined submissions for both MS and MSMS were Acetyl (K), Acetyl (N-term), Carbamidomethyl (C), Oxidation (M), Deamidated (NQ), Phospho (ST), Phospho (Y).
**Depot-specific protein content**

Protein concentration data measured in the samples treated for proteomics (see above) were used to estimate the protein content in each of the depots. The calculation consisted of multiplying the volume of protein solution obtained (in milliliters) by the concentration of isolated proteins (in milligrams per milliliter) to obtain the total weight of protein isolated (in milligrams). This value was then divided by the initial weight of the processed sample (in grams). The results are expressed in milligrams of protein per gram of tissue.

**Histology and adipocyte sizing**

Formalin-fixed samples were embedded in paraffin, cut into 5-µm sections, mounted, and stained with hematoxylin and eosin. Stained slides were examined with a Nikon Eclipse E600 microscope equipped with a SPOT RT (Real-time) digital camera and using a 20X objective. Digital images were taken for three non-overlapped microscope fields in each WAT depot from each animal. Cell size (cross-sectional area) was determined as described by Chen and Farese (2002). For each mouse and at each WAT depot, the mean adipocyte size was calculated based on the cross-sectional area of all the adipocytes in the three non-overlapped fields.

**Statistical analysis**

Spot intensity data were log-transformed, and all data (WAT depot weight, protein content, mean adipocyte cross-sectional area, log-transformed spot intensity, and
plasma hormone levels) were checked for normality using Shapiro-Wilk’s test ($P<0.05$). When the data followed a normal distribution, means were compared among depots using a within-subjects one-way ANOVA. Sphericity was tested using Mauchly’s method, and a Greenhouse-Geisser correction was applied when the assumption of sphericity was not met ($P<0.05$). Data displaying non-normal distributions were analyzed using Friedman’s non-parametric test. When significant differences were found among depots, post-hoc tests were performed. Tukey’s HSD test was applied to normally distributed data and the Bonferroni-Dunn method was applied to the rest. Pearson correlations were evaluated on normally-distributed data and Spearman’s method was used for non-normal distributions.

Statistical significance cutoffs were $P<0.01$ for spot intensity differences among depots (given the intrinsic variability of the 2DE technique) and $P<0.05$ for comparisons of WAT depot weights, protein concentrations, mean adipocyte cross-sectional areas, and for all correlations. The software used was SPSS v14.0; post-hoc tests were performed manually in Excel spreadsheets.

Results

Body weight, body composition, and plasma hormone levels

Table 7 summarizes the characteristics of the mice studied, including body weight, body composition, and fasting plasma levels of insulin, leptin, and total and HMW adiponectin. The HMW form of adiponectin recently has gained attention in terms of its ability to act as an insulin sensitizer, as it has been suggested to represent the active form of adiponectin (Kaser et al., 2008; Waki et al., 2003; Whitehead et al., 2006). In
humans, only HMW adiponectin decreases in obesity, while the other adiponectin isoforms remain constant (Kaser et al., 2008). In this study, levels of HMW adiponectin correlated closely to total adiponectin levels ($r^2=0.89$, $n=10$, $P<0.001$, Figure 17).

**Figure 17.** Correlation between HMW and total adiponectin plasma levels in 12-month-old WT mice ($n=10$).
## Table 7

**Characteristics of 12-Month-Old WT Mice**

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Fat mass (%</th>
<th>Lean mass (%)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Total Adip (µg/ml)</th>
<th>HMW Adip (µg/ml)</th>
<th>WAT depot weight (mg)</th>
<th>Protein content (mg/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>38.8</td>
<td>25.3</td>
<td>64.5</td>
<td>3.16</td>
<td>31.4</td>
<td>29.1</td>
<td>10.6</td>
<td>881&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE</td>
<td>1.0</td>
<td>1.6</td>
<td>1.2</td>
<td>0.58</td>
<td>5.1</td>
<td>2.0</td>
<td>1.4</td>
<td>106&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All mice were male and 12-month-old. *n*=10 unless otherwise noted. Different superscripts or symbols denote significant differences (*P*<0.05) as determined in a Friedman test for depot weights and a within-subjects one-way ANOVA for protein content. Abbreviations: Adip, adiponectin; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.
Depot-specific weight and protein content

WAT from four depots was weighed at dissection. The epididymal depot was significantly larger and the retroperitoneal depot significantly smaller than the others \((P<0.001, \text{Table 7})\). Samples were homogenized, their protein concentration determined and the protein content per gram of tissue calculated (see Materials and Methods). The mesenteric depot displayed significantly higher levels of protein per gram of tissue than the others \((P=0.002, \text{Table 7})\).

Correlations of hormone levels with total and depot-specific fat masses

We evaluated correlations between all the measured hormones and total and depot-specific fat masses. Leptin levels correlated positively with total fat mass \((r^2=0.77, n=10, P<0.001)\) and fat mass percentage \((r^2=0.73, n=10, P=0.002)\) (Figure 18). On the other hand, there was no significant correlation of total or HMW adiponectin or insulin levels with total fat or any WAT depot-specific masses.
Figure 18. Correlation between plasma leptin levels and fat mass or percent fat mass in 12-month-old WT mice (n=10).

Depot-specific proteomes and protein identification by mass spectrometry

Proteomic profiles of the four WAT depots were determined by 2DE. We compared the intensities of 166 spots, 38 of which showed significant differences among WAT depots (P<0.01, Figures 19 to 22). Using MS and MSMS, 15 unique proteins were identified, with many spots representing isoforms of the same protein (Table 8). Overall, isoforms of the same protein tended to display similar trends in the four WAT depots (Figures 21 and 22). These isoforms probably result from post-translational modifications, which generate subtle changes in the pI and/or molecular weight of the proteins. Table 9 summarizes the proteins identified, their intensities in the WAT depots, and their functions (generation of ATP, glucose and lipid metabolism, lipid transport, stress resistance, cytoskeleton structure, etc.).
Figure 19. Representative 2D-gel of WAT. A. Raw image. B. Same gel image showing 38 spots (x) that displayed significant intensity differences among depots ($P<0.01$). The dotted square delineates the gel area shown in Figure 20. Numbered labels correspond to protein identities shown in Table 8.
Figure 20. Topographical representation of the intensity of an individual spot (9a) in different WAT depots of the same mouse. Images were obtained using the 3D-viewer tool of PDQuest Advanced v8.0 (Bio-Rad) after normalization to total density in each gel. The gel area shown corresponds to the dotted square delineated in Figure 19. Spot 9a was selected as a representative spot that displays intensity variations among WAT depots.
Figure 21. Box plots of log-transformed spot intensity for 20 spots that were significantly different among WAT depots in 12-month-old WT mice (data from six different mice).
† indicates significant difference ($P<0.01$). Protein spots are organized according to their functions: A. ATP generation; B. Glucose/lipid metabolism; C. Lipid transport; D. Stress-resistance. The remaining 18 significant spots have been grouped in two other categories (E. Cytoskeleton; F. Other) and are plotted in Figure 22. Protein identities were obtained by MS and MSMS (Table 8). Abbreviations: CKB, creatine kinase B; ATP5H, ATP synthase subunit d; TIM, triosephosphate isomerase; ENO, enolase; CA-III, carbonic anhydrase 3; APOA-I, apolipoprotein A-I; AFP, α-fetoprotein; HSC70, heat shock cognate 71 kDa protein; HSPβ1, heat shock protein β1; PRDX2, peroxiredoxin 2.
Figure 22. Box plots of log-transformed spot intensity for 18 spots that were significantly different among WAT depots in 12-month-old WT mice (data from six different mice). † indicates significant difference ($P<0.01$). Spots 11 and 14 were only present in mesenteric fat. Protein spots are organized according to their functions: E. Cytoskeleton; F. Other. The remaining 20 significant spots have been grouped in four other categories (A. ATP generation; B. Glucose/lipid metabolism; C. Lipid transport; D. Stress-resistance) and are plotted in Figure 21. Protein identities were obtained by MS and MSMS (Table 8). Abbreviations: MYL9, myosin regulatory light polypeptide 9; Hb-β, hemoglobin-β.
Table 8

**MS and MSMS Matches for Spots Displaying Significant Intensity Differences among WAT Depots in 12-Month-Old WT mice**

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<th>Spot</th>
<th>Accession</th>
<th>Protein name</th>
<th>Unique peptides detected</th>
<th>Sequence coverage (%)</th>
<th>Score or CI %*</th>
<th>MSMS peptides matched</th>
<th>Error (Da)</th>
<th>m/z Score</th>
<th>Score</th>
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<tr>
<td>1</td>
<td>P63017</td>
<td>Heat shock cognate 71 kDa protein (HSC70, heat shock 70 kDa protein 8)</td>
<td>8</td>
<td>19</td>
<td>86</td>
<td>5</td>
<td>R.ARFEELNADLFRC.G</td>
<td>0.0750</td>
<td>1480.829</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K.TVTNAVVTVPAYFN</td>
<td>0.0778</td>
<td>1982.076</td>
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<td></td>
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<td></td>
<td></td>
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<td>2a</td>
<td>Q04447</td>
<td>Creatine kinase B-type (CKB)</td>
<td>3</td>
<td>10</td>
<td>100%</td>
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<th>Sequence coverage (%)</th>
<th>Score or CI %*</th>
<th>MSMS peptides matched</th>
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Note: The Table provides information about the unique peptides detected, their sequence coverage, score or CI%, MSMS peptides matched, and the error in Da. It also includes the m/z and score for each entry.
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Although the NCBInr database was searched, accession numbers listed are from the SWISS-Prot database, so as to unify in an individual entry all the possible matches representing sequence variants of the same protein. (*) Spots identified via manual online searches on Mascot display MS scores; spots identified using the GPS Explorer show confidence intervals (CI %). Peptide mass fingerprint (MS) scores above 64 and confidence intervals (CI%) above 95% are significant \((P<0.05)\) and are shown in bold. Peptide MSMS scores obtained from manual searches on Mascot are shown as \([\text{score/significance cutoff}]\). Significant \((P<0.05)\) peptide MSMS scores are also shown in bold. See materials and methods for a detailed description of the methodology used. (**) For most of the spots labeled “5” only the best scoring isoforms of actin are reported; however, more isoforms were matched with slightly lower scores. (***) Low quality MSMS data. (†) Peptides and fragments matching CA-III probably represent contamination by surrounding spots, given that the region of the gel in and around spot 8 contains significant amounts of CA-III isoforms. (††). The quality of the peptides and fragment matching ferritin light chain 1 was not enough to confidently assign this protein identity to spot 13a. However,
the SWISS-2DPAGE database (Hoogland et al., 2004) shows a similar gel location for this protein. Therefore, the presence of ferritin light chain 1 in spot 13a could not be discarded.

Table 9

Summary of Protein Identities, Depot-Specific Intensities and Protein Function for Spots Displaying Significant Intensity Differences among WAT Depots in 12-Month-Old WT mice (P<0.01)

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<td></td>
<td>Inguinal Retroperitoneal Mesenteric Epididymal</td>
<td></td>
</tr>
<tr>
<td>ATP generation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a-c</td>
<td>Creatine kinase B-type</td>
<td>+ / ++</td>
<td>+ / ++</td>
</tr>
<tr>
<td>13a-b</td>
<td>ATP synthase subunit d, mitochondrial (might also contain a ferritin light chain)</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 9: continued

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Relative intensity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inguinal</td>
<td>Retroperitoneal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++ / +++</td>
<td>++ / +++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Glucose/lipid metabolism

8 Triosephosphate isomerase (might also contain CA-KIII)

6 Enolase

7a-d Carbonic anhydrase 3 (CA-III)

Lipid transport

10a-c Apolipoprotein A-I (ApoA-I)

3 α-fetoprotein
<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Relative intensity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inguinal</td>
<td>Retroperitoneal</td>
</tr>
<tr>
<td>Stress resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Heat shock cognate 71 kDa protein (HSC70, heat shock 70 kDa protein 8)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9a-b</td>
<td>Heat shock protein β1 (HSPβ1, heat shock 27 kDa protein, HSP27)</td>
<td>+</td>
<td>++ / +++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12a-b</td>
<td>Peroxiredoxin-2 (thioredoxin peroxidase 1)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Actin (various forms)</td>
<td>+ / ++</td>
<td>+ / ++</td>
</tr>
<tr>
<td>5a-k</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot</td>
<td>Protein identity</td>
<td>Relative intensity</td>
<td>Function</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>--------------------</td>
<td>----------</td>
</tr>
<tr>
<td>11</td>
<td>Myosin regulatory light polypeptide 9 (MYL9, myosin regulatory light chain 2, smooth muscle isoform)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>14</td>
<td>Transgelin (smooth muscle protein 22-α)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>15a-c</td>
<td>Hemoglobin subunit β-1</td>
<td>+</td>
<td>+/++ / +++</td>
</tr>
<tr>
<td>4a-b</td>
<td>14-3-3 protein γ</td>
<td>++</td>
<td>++/ +++</td>
</tr>
</tbody>
</table>

Protein identities were obtained by MS and MSMS (Table 8). Spot labels correspond to the gel locations shown in Figure 19. As way of summary, the intensities of isoforms of a same protein were grouped into a single value using a qualitative scale with (+) signs. Intensity values marked “+” (low) are significantly different from “+++” (high). Intensities marked “++” are intermediate and not
significantly different from “+” (low) or “+++” (high). Grouping of isoform intensities was possible as they displayed similar trends among WAT depots. Two exceptions were observed among isoforms of CA-III (7a-d) and of hemoglobin subunit β-1 (15a-c) where intensity values ranged from low (+) to high (+++) in the mesenteric and retroperitoneal depots, respectively (see Figures 21 and 22).
Correlations between spot intensity and circulating hormone levels

When evaluating possible correlations between the intensities of the 38 spots that changed significantly among WAT depots and the levels of plasma hormones measured, isoform- and depot-specific correlations were found for some proteins (Table 10). There were evident overlaps in the proteins that correlated to insulin and those that showed significant correlations to leptin, HMW and total adiponectin, which is noteworthy given that insulin levels did not show correlations with the levels of other hormones. Some of these overlaps, however, involved correlations present in different depots or for different isoforms of the same protein. For example, insulin levels correlated negatively with triosephosphate isomerase (TIM) levels in the retroperitoneal depot, whereas leptin and total adiponectin correlated negatively with TIM levels in the epididymal depot (Table 10). Also interesting was the behavior of different actin isoforms, which displayed opposite correlations (positive vs. negative) to a particular hormone (as is the case for insulin and total adiponectin).
Table 10

*Significant Correlations between Circulating Hormone Levels and Spot Intensities in 12-Month-Old WT Mice*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CKB</td>
<td>2b</td>
<td>Epi</td>
<td>$r^2=0.75; P=0.026$</td>
<td>TIM</td>
<td>8</td>
<td>Ret</td>
<td>$r^2=0.72; P=0.033$</td>
</tr>
<tr>
<td>CKB</td>
<td>2c</td>
<td>Epi</td>
<td>$r^2=0.81; P=0.015$</td>
<td>ApoA-I</td>
<td>10a</td>
<td>Ret</td>
<td>$r^2=0.71; P=0.035$</td>
</tr>
<tr>
<td>CA-III</td>
<td>7d</td>
<td>Ing</td>
<td>$r^2=0.78; P=0.020$</td>
<td>ApoA-I</td>
<td>10a</td>
<td>Epi</td>
<td>$r^2=0.81; P=0.014$</td>
</tr>
<tr>
<td>Actin</td>
<td>5h</td>
<td>Epi</td>
<td>$r^2=0.73; P=0.031$</td>
<td>ApoA-I</td>
<td>10b</td>
<td>Ing</td>
<td>$r^2=0.67; P=0.047$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HSPβ1</td>
<td>9b</td>
<td>Ing</td>
<td>$r^2=0.76; P=0.023$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HSPβ1</td>
<td>9b</td>
<td>Epi</td>
<td>$r^2=0.68; P=0.043$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actin</td>
<td>5b</td>
<td>Mes</td>
<td>$r^2=0.76; P=0.023$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actin</td>
<td>5c</td>
<td>Epi</td>
<td>$r^2=0.75; P=0.026$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actin</td>
<td>5k</td>
<td>Mes</td>
<td>$r^2=0.74; P=0.028$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Transgelin</td>
<td>14</td>
<td>Mes</td>
<td>$r^2=0.88; P=0.005$</td>
</tr>
</tbody>
</table>
### Table 10: continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA-III</td>
<td>7b</td>
<td>Ret</td>
<td>$r^2=0.89; P=0.005$</td>
<td>TIM</td>
<td>8</td>
<td>Epi</td>
<td>$r^2=0.77; P=0.022$</td>
</tr>
<tr>
<td>Actin</td>
<td>5b</td>
<td>Epi</td>
<td>$r^2=0.82; P=0.013$</td>
<td>ApoA-I</td>
<td>10a</td>
<td>Epi</td>
<td>$r^2=0.70; P=0.037$</td>
</tr>
<tr>
<td>Actin</td>
<td>5g</td>
<td>Ing</td>
<td>$r^2=0.71; P=0.034$</td>
<td>$\alpha$-fetoprotein</td>
<td>3</td>
<td>Mes</td>
<td>$r^2=0.73; P=0.031$</td>
</tr>
<tr>
<td>Actin</td>
<td>5i</td>
<td>Ing</td>
<td>$r^2=0.75; P=0.026$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>5i</td>
<td>Ret</td>
<td>$r^2=0.70; P=0.038$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total adiponectin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA-III</td>
<td>7b</td>
<td>Ret</td>
<td>$r^2=0.88; P=0.006$</td>
<td>TIM</td>
<td>8</td>
<td>Epi</td>
<td>$r^2=0.72; P=0.032$</td>
</tr>
<tr>
<td>Actin</td>
<td>5i</td>
<td>Ing</td>
<td>$r^2=0.78; P=0.020$</td>
<td>$\alpha$-fetoprotein</td>
<td>3</td>
<td>Mes</td>
<td>$r^2=0.70; P=0.037$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actin</td>
<td>5b</td>
<td>Mes</td>
<td>$r^2=0.89; P=0.005$</td>
</tr>
<tr>
<td><strong>HMW adiponectin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CKB</td>
<td>2c</td>
<td>Epi</td>
<td>$r^2=0.82; P=0.014$</td>
<td>ApoA-I</td>
<td>10a</td>
<td>Epi</td>
<td>$r^2=0.86; P=0.008$</td>
</tr>
</tbody>
</table>
Table 10: continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot</th>
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<th>Protein</th>
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<th>Depot</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-III</td>
<td>7b</td>
<td>Ret</td>
<td>$r^2=0.68; P=0.043$</td>
<td>Actin</td>
<td>5b</td>
<td>Mes</td>
<td>$r^2=0.91; P=0.003$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actin</td>
<td>5k</td>
<td>Mes</td>
<td>$r^2=0.77; P=0.021$</td>
</tr>
<tr>
<td>Transgelin</td>
<td>14</td>
<td>Mes</td>
<td>$r^2=0.70; P=0.039$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results correspond to $r^2$ and $P$ values for Pearson correlation tests. Abbreviations: CKB, creatine kinase B; CA-III, carbonic anhydrase 3; TIM, triosephosphate isomerase; ApoA-I, apolipoprotein A-I; HSPβ1, heat shock protein β1; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.
Adipocyte size

Given that for some proteins, expression in adipocytes is influenced by adipocyte size (as reported for LPL, HSL, leptin (Vidal, 2001) and many pro- and anti-inflammatory factors (Skurk et al., 2007), see Chapter 1), adipocyte cross-sectional areas were measured in the four WAT depots studied (Figure 23). Mean adipocyte sizes of the inguinal and mesenteric depots were significantly smaller than that of the epididymal depot ($P=0.003$, Figure 23C). The increased mean adipocyte cross-sectional area in epididymal fat resulted from a relative increase in the number of large adipocytes ($>8,000 \mu m^2$) and a relative decrease in the number of small adipocytes ($<4,000 \mu m^2$) (Figure 23A, B). The retroperitoneal depot displayed high amounts of large adipocytes as well, but also moderate amounts of small adipocytes (Figure 23A, B). Therefore, the mean adipocyte cross-sectional area in retroperitoneal fat showed a trend towards increase, but was not significantly higher than in the inguinal and mesenteric WAT depots (Figure 23C). Along the same line, high numbers of small adipocytes in inguinal and mesenteric fat resulted in significantly smaller mean adipocyte cross-sectional areas in these WAT depots. Interestingly, one mouse showed markedly increased mean adipocyte sizes in inguinal and mesenteric WAT, which represented outliers in our sample’s distribution (Figure 23C). This mouse had the highest fat mass percentage, largest WAT depots, and highest leptin levels of the group as well. However, epididymal and retroperitoneal mean adipocyte sizes in this mouse were similar to those of the other mice studied. Also, levels of insulin, total and HMW adiponectin were intermediate among the mice in the group.
For these reasons, and given that we regularly encounter similar “overweight” mice in the C57 background (unpublished observations), data from this mouse were not discarded.

![Depot-specific adipocyte sizes in 12-month-old WT mice. A. Hematoxylin and eosin-stained slides of paraffin-embedded samples were examined using a 20X objective. Cell size was determined as cross-sectional area. Scale bar = 50 µm. B. Histograms of adipocyte sizes in the four WAT depots (areas measured in six different mice are grouped in each graph). C. Mean adipocyte size for each WAT depot (n=6). Different letters indicate significant differences in Friedman’s test (P=0.003).]

Figure 23. Depot-specific adipocyte sizes in 12-month-old WT mice. A. Hematoxylin and eosin-stained slides of paraffin-embedded samples were examined using a 20X objective. Cell size was determined as cross-sectional area. Scale bar = 50 µm. B. Histograms of adipocyte sizes in the four WAT depots (areas measured in six different mice are grouped in each graph). C. Mean adipocyte size for each WAT depot (n=6). Different letters indicate significant differences in Friedman’s test (P=0.003).

Blood samples were available for only four of the six mice assigned to histological analysis. Interestingly, despite the low number of samples, a significant positive correlation was found between total adiponectin levels and the mean adipocyte
size of the epididymal WAT depot ($r^2=0.99, n=4, P=0.008$) (Figure 24A). In addition, mean adipocyte size in mesenteric fat correlated significantly to mesenteric fat mass ($r^2=0.75, n=6, P=0.025$) (Figure 24B). This indicates that increases in mesenteric fat could reflect increased adipocyte size rather than number, which is consistent with observations performed in middle-aged men (Marin et al., 1992a).

![Figure 24](image-url)

*Figure 24.* Relationships between mean adipocyte sizes and plasma hormone levels or fat masses of 12-month-old WT mice. A. Correlation between epididymal mean adipocyte size and circulating adiponectin levels (n=4). B. Correlation between mesenteric mean adipocyte size and mesenteric and total fat mass (n=10).

When correlations between the intensity of each spot and mean adipocyte size in each depot were tested, no significant relations were found. Given that the proteomic analysis was performed on one group of mice and that histology slides were obtained using a different group, these correlations could not be tested on individual mouse data. In contrast, they were tested using the mean values in each mouse group. Therefore, only
four values for mean adipocyte size and four values for each spot’s intensity mean (one per depot) were used to test this correlation. The decreased sample size might be the reason why none of the spot intensities correlated to mean adipocyte sizes in the depots.

Discussion

This study analyzed the proteomic and main phenotypic differences among four WAT depots of healthy 12-month-old WT C57Bl/6J mice. Given that the C57Bl/6J strain is commonly used in mouse studies of obesity and diabetes, it is of particular importance to understand the characteristics of WAT in these mice. Furthermore, whole tissue was analyzed (as opposed to isolated adipocytes), for a more comprehensive analysis of the characteristics of WAT in vivo. In our samples, the corresponding mixture of cell populations, the tissue architecture and the microenvironment were unscathed. As expected, several differences among the four WAT depots were found, including depot weight, total protein content, mean adipocyte size, and expression profiles of numerous proteins. The recorded weights for inguinal, retroperitoneal, mesenteric and epididymal WAT depots were consistent with those reported recently by Palmer et al. (2009). Interestingly, the mesenteric WAT depot showed protein content per gram of tissue that was twice as high as the other three depots. This is probably due to the higher amount of blood vessels and lymph nodes present in mesenteric WAT. In fact, higher blood flow in mesenteric compared to retroperitoneal, inguinal and epididymal WAT depots was previously described for rats (Crandall et al., 1984).
Plasma levels of insulin, leptin and adiponectin (total and HMW) were analyzed for correlations to total and depot-specific fat masses. Consistent with our data, leptin levels are normally positively correlated to body fat both in humans and mice (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1995). On the other hand, neither total nor HMW adiponectin showed significant correlations with fat mass or any of the fat depot masses recorded. Total and HMW adiponectin levels decrease in obese individuals (Kaser et al., 2008), nevertheless, this negative association possibly develops only when changes in fat mass are drastic or when insulin sensitivity is affected, as seen in ob/ob and db/db mice (Hu et al., 1996; Yamauchi et al., 2001). Similarly, the overall health and normal weight of the mice analyzed in our study might account for the lack of correlation between insulin and fat mass, fat mass percentage and depot-specific fat masses.

Mean adipocyte size was largest in epididymal WAT and smallest in the inguinal and mesenteric depots. These results also are consistent with previous reports. In mice, the mean adipocyte size in the inguinal WAT depot was significantly smaller than in epididymal and retroperitoneal fat (Kelder et al., 2007; Zhang et al., 2002). In rats, inguinal and mesenteric adipocytes were smaller than cells in retroperitoneal fat (Palou et al., 2009). Also, Matsubara et al. (2009) recently found adipocyte diameter distributions to be almost identical when comparing adipocytes from inguinal fat to those of pooled mesenteric and perirenal WAT of, C57BL/6J mice.

We also found a positive correlation between total plasma adiponectin levels and epididymal mean adipocyte size. Adiponectin secretion and mRNA levels have been shown to correlate positively to cell size in cultured adipocytes (Skurk et al., 2007).
However, plasma adiponectin shows a negative correlation to adipocyte size in the omental WAT depot of obese middle-aged women (Drolet et al., 2009). The difference between the latter study and our results may be due to the normal weight of the mice we examined, although a difference between species cannot be discarded (especially given the differences in relative sizes of adipocytes in individual depots of humans and rodents described in Chapter 2).

When the proteomes of the four WAT depots were resolved by 2DE, 15 protein identities were found to vary among depots. Two of these were enzymes related to ATP generation (CK type B (CKB, three isoforms) and ATP synthase subunit d (two isoforms)), which were increased in mesenteric WAT. As reviewed in Chapter 1, in humans, VISC WAT (equivalent to mouse mesenteric and partly to epididymal WAT, Chapter 2) displays higher rates of TG turnover than SC fat (Marin et al., 1992a). This may account for a higher energetic need in mesenteric WAT and consequently higher ATP synthesis and phosphate transfer to creatine, catalyzed by ATP synthase and CKB respectively. In 1976, Berlet et al. (1976) proposed that energy metabolism in AT depended closely on creatine, after demonstrating CK activity in WAT and BAT of mice. Differences in CK activity among abdominal SC, thigh SC, epididymal fat and fat lining the spleen were reported previously for rats (Somjen et al., 1997). Consistent with our results, epididymal and thigh SC WAT had similar CK activity (Somjen et al., 1997). Given that insulin signaling requires ATP for both its lipogenic and antilipolytic effects, the energy status of adipocytes may have critical implications on insulin sensitivity (reviewed in Kopecky et al., 2002). In fact, in this study, isoforms of CKB in specific
depots were found to correlate positively to circulating insulin levels (Table 10). Recently, however, immunoreactive CKB was found only in nerves and blood vessels of AT and not in adipocytes (Streijger et al., 2009). Therefore, elevated CKB levels in mesenteric WAT may reflect high density of blood vessels and nerves in this depot.

In adipocytes, glucose oxidation via glycolysis, Krebs cycle and oxidative phosphorylation provides the cell with ATP and carbon substrates for de novo fatty acid synthesis and TG synthesis. Glucose entry to the cell through the GLUT4 transporter is regulated by insulin, hence WAT depots that are more insulin sensitive may display enhanced glucose metabolism. In our study, two glycolytic enzymes showed differential expression in the WAT depots analyzed. TIM catalyzes the reversible interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) in the fifth step of glycolysis; enolase (ENO) interconverts 2-phosphoglycerate (2PG) and phosphoenolpyruvate (PEP) in the ninth step of the pathway (Figure 25). On the other hand, glyceroneogenesis is also a major pathway in adipocyte metabolism, providing G3P for fatty acid re-esterification upon lipolysis (Nye et al., 2008). Glyceroneogenesis, a commonly overlooked pathway that involves the synthesis of G3P from precursors other than glucose or glycerol, was first discovered in the 1960s (Ballard et al., 1967). This pathway is a shortened version of gluconeogenesis, and involves the conversion of pyruvate to DHAP, which is subsequently reduced to G3P (Figure 25). Re-esterification of FFA with G3P decreases fatty acid release from WAT during lipolysis (Figure 26). This process, by decreasing FFA release to the bloodstream, has been suggested to decrease lipotoxicity and improve insulin sensitivity, and to be the main mechanism of
action of the antidiabetic drugs thiazolidinediones (TZDs) (Cadoudal et al., 2005; Tordjman et al., 2003). Both TIM and ENO take part in glyceroneogenesis, and the cytosolic isoform of PEPCK (PEPCK-C) is the rate limiting enzyme of the pathway.

*Figure 25.* Schematic representation of glycolysis and glyceroneogenesis in WAT. Glycolytic enzymes shown in red displayed significant intensity differences among the depots analyzed. Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; pyr, pyruvate; TG, triglycerides.
Figure 26. Glyceroneogenesis provides glycerol-3-phosphate for the re-esterification of fatty acids upon activation of lipolysis. This is expected to decrease fatty acid release to the bloodstream. Abbreviations: TG, triglycerides.

Whether the levels of TIM and ENO detected in this study reflect their activity in glycolysis or glyceroneogenesis remains uncertain. Overall, our results showed that TIM and ENO levels were highest in epididymal fat, suggesting that glycolysis, glyceroneogenesis, or both are activated in this WAT depot. Others (Festuccia et al., 2009; Tordjman et al., 2003) have also found glyceroneogenesis and glucose oxidation rates in rats to be higher in perirenal, omental and retroperitoneal WAT depots than inguinal fat. In addition, levels of TIM are higher in omental than SC WAT in obese humans (Perez-Perez et al., 2009), consistent with our results showing higher TIM levels in epididymal than inguinal fat in mice. Given that the rate of TG turnover is generally higher in intra-abdominal (omental) than SC fat (Marin et al., 1992a), it seems appropriate that glyceroneogenesis in epididymal WAT would be relatively increased. In fact, lower protein levels of IRS-1 and mRNA levels of GLUT4 and glycogen synthase in
human omental WAT point to lower insulin sensitivity in this depot (Lefebvre et al., 1998; Zierath et al., 1998), which is consistent with lower inhibition of PEPCK-C, leading to higher glyceroneogenesis. Thus, our data suggests that the epididymal fat depot could be central to regulating the impact of TZDs in the body. Further studies differentiating the activation status of the glycolytic and glyceroneogenic pathways in different WAT depots will provide broader insight into this matter. Recently, Nye et al. (2008) proposed that primarily glycolytic cells in WAT, such as endothelial cells, macrophages and preadipocytes, might oxidize glucose to lactate and thus provide this substrate for glyceroneogenesis and TG synthesis in fat accumulating adipocytes. If this were the case, different proportions of cell populations in WAT depots could explain the dissimilar enzyme levels observed in this study.

Four isoforms of carbonic anhydrase 3 (CA-III) were differentially expressed among WAT depots. Although the physiological function of this enzyme in AT is not well understood, a role in intracellular pH regulation upon lipolysis and in the conversion of pyruvate to G3P and fatty acids has been proposed (Coulson & Herbert, 1984). CA provides bicarbonate for carbon-fixing reactions such as the carboxylation of pyruvate to oxaloacetate for gluco/glyceroneogenesis and that of acetyl-CoA to malonyl-CoA for fatty acid synthesis (Coulson & Herbert, 1984; Herbert & Coulson, 1984; Herbert et al., 1983) (Figure 25). Consistent with this function, CA-III is expressed only after differentiation in 3T3-L1 adipocytes, when cells start to accumulate lipid (Lynch et al., 1993). Our data showed low levels of CA-III in inguinal fat, which are consistent with lower TG turnover in this depot.
The levels of three isoforms of ApoA-I were high in retroperitoneal and mesenteric WAT and low in inguinal and epididymal WAT. Given that ApoA-I is the major protein in HDL-cholesterol, differential expression of ApoA-I could indicate that the rate of cholesterol transport from WAT to the liver is depot-dependent. Anti-inflammatory functions have also been proposed for ApoA-I and HDL (Barter et al., 2007). Thus, the different levels of ApoA-I might affect the expression of endothelial adhesion molecules in each depot. The ApoA-I levels measured most probably represent HDL particles bound to adipocyte membranes in the dissected tissues (Fong et al., 1986), which might depend on tissue blood flow. In fact, the depot-specific trends of ApoA-I isoforms follow a similar pattern to those of hemoglobin-β isoforms. It is not clear, however, why levels of isoforms of hemoglobin-β and ApoA-I are relatively high in retroperitoneal fat, given that blood flow in this depot is similar to epididymal and inguinal WAT (Crandall et al., 1984). On the other hand, even though ApoA-I is mainly synthesized by the liver and small intestine (The UniProt Consortium, 2009), mRNA expression of this and other apolipoproteins also has been detected in WAT from humans, pigs and mice (Castro-Chavez et al., 2003; Hausman et al., 2007; Hausman et al., 2006; Maeda et al., 1997; Nadler et al., 2000; Ross et al., 2002). Therefore, rather than the circulation, WAT itself cannot be ruled out as the source of the measured ApoA-I in this study. If that were the case, synthesis of ApoA-I would appear to be regulated differentially in the four WAT depots. Moreover, as with apolipoproteins, hemoglobin-β expression also has been found in microarray analyses of WAT in humans and mice (Castro-Chavez et al., 2003; Gabrielsson et al., 2000; Nadler et al., 2000). Another
protein related to lipid transport, α-fetoprotein, was increased in the mesenteric depot. Apart from FFA, α-fetoprotein is known to transport estrogens and metals in the bloodstream (The UniProt Consortium, 2009). Because this protein is present in the circulation, and given that mesenteric fat displays high blood flow, high levels of α-fetoprotein in mesenteric fat might represent circulating levels of this protein.

Three proteins related to cell stress were differentially regulated among WAT depots. Heat shock cognate 71 kDa protein (HSC70) is a chaperone of the HSP 70 family and is found in the cytoplasm forming part of a mRNP granule complex with various proteins and untranslated mRNAs (Jonson et al., 2007; The UniProt Consortium, 2009). H. S. Chen et al. (2006) reported an increase in HSC70 protein levels in WAT of type 1 diabetic rats that was normalized by insulin treatment. However, more information is necessary to characterize the responsiveness of HSC70 to insulin and other stimuli in the different WAT depots. Our data show high HSC70 levels in inguinal and epididymal WAT (suggesting high chaperone activity in these depots) and low levels in mesenteric fat, which might be more susceptible to stress from misfolded proteins. Heat shock protein β1 (HSPβ1) is involved in stress resistance and actin organization and a member of the HSP 20 family. This protein protects the cell against heat shock, oxidative stress and chemotherapy drugs (Arrigo, 2001). Also, HSPβ1 has been shown to mediate pro-inflammatory cell signaling in response to interleukin-1 (IL-1) and TNF (Alford et al., 2007). HSPβ1 was low in inguinal and higher in the remaining depots. Similarly, in human obese subjects HSPβ1 was higher in omental than abdominal SC WAT (Perez-Perez et al., 2009). This is consistent with intra-abdominal depots displaying enhanced
responsiveness to inflammatory signals than SC WAT depots (Vohl et al., 2004). It could also be related to the increased metabolism in intra-abdominal WAT that may generate more ROS and might therefore need enhanced defense against oxidative damage.

Peroxiredoxin 2 (PRDX2) is also a chaperone and an antioxidant protein that degrades H$_2$O$_2$ and organic peroxides, including lipid peroxides (Low et al., 2008). In addition, this enzyme may affect signaling pathways in which H$_2$O$_2$ is a secondary messenger, such as that of TNF-α (Kang et al., 2004). PRDX2 levels were highest in mesenteric fat and lowest in inguinal WAT. Again, the higher rate of lipolysis and enhanced immune responsiveness of the intra-abdominal WAT depots might explain these differences. However, PRDX2 is highly abundant in erythrocytes (Low et al., 2008), so the intensity changes might reflect differences in depot blood flow.

Differences were also found in 11 isoforms of actin. Unfortunately, discrimination between α-, β- or γ-forms of actin was not possible with the mass spectrometric data obtained (Table 8). We suspect that the increased levels of actin in the mesenteric WAT depot correspond, at least in part, to α- and γ-actin from smooth muscle in blood vessel walls. Also, mesenteric WAT was the only depot that displayed spots containing transgelin and myosin regulatory light polypeptide 9 (MYL9). Expression of MYL9 (along with other muscle-associated proteins) was detected also in BAT of PPARα-null mice, suggesting that PPARα normally suppresses expression of MYL9 at least in BAT (Tong et al., 2005). To our knowledge there are no reports on MYL9 expression in WAT. Because MYL9 and transgelin are known constituents of smooth muscle (Kumar et al.,
1989; Solway et al., 1995), the presence of these proteins in mesenteric WAT emphasizes the increased density of blood vessels in this fat depot as compared to the others.

Finally, two isoforms of 14-3-3γ showed differential expression among WAT depots. The signaling molecule 14-3-3γ is involved in many intracellular pathways, such as the PI3K/Akt pathway and the MAPK pathway, making it difficult to interpret the intensity differences found for this protein.

Adipocyte size is another factor influencing protein expression in WAT, as has been reported for lipoprotein lipase, hormone-sensitive lipase and leptin (Vidal, 2001) and many pro- and anti-inflammatory factors (Skurk et al., 2007). However, in this study mean spot intensities in each depot did not correlate significantly to mean adipocyte sizes.

In summary, the present characterization of WAT in 12-month-old WT mice clearly demonstrates marked differences among depots in the expression of several proteins and in protein content and adipocyte sizes. The main characteristics observed in this study for each WAT depot are summarized in Figure 27. A few of the variations among depots were consistent with larger blood supply and higher blood vessel density in the mesenteric depot. Also, the proteome of the inguinal depot showed major differences from those of mesenteric and epididymal WAT. The expression of enzymes involved in the generation of ATP, glycolysis/glyceroneogenesis, fatty acid synthesis, and antioxidant proteins were lower in inguinal WAT. These results are consistent with slower TG turnover and consequently lower ROS production in inguinal as compared to mesenteric and epididymal WAT. Thus, overall metabolic rate and stress are probably high in
mesenteric and epididymal WAT and low in inguinal WAT, with intermediate levels in the retroperitoneal depot. Together, this information enhances the understanding of WAT physiology and the role of different WAT depots in metabolism and homeostasis. The differences observed in protein expression patterns among WAT depots should fuel the search for depot-specific diagnostic and therapeutic targets of obesity-related diseases, including metabolic syndrome, type 2 diabetes, cardiovascular disease, and cancer.

**Figure 27.** Location and main characteristics of the WAT depots studied. Epididymal depots have been moved outside of the abdomen while conserving their attachment point. Inset image shows the abdomen after dissection of the intestines, to view the kidney (K) and the retroperitoneal depot.
Acknowledgements

Special thanks to Rachel D Munn for doing the tedious technical work required to measure adipocyte cross-sectional areas, Ellen R Lubbers for performing the adiponectin (total and HMW) and insulin ELISAS, Dr. Darlene Berryman for running the leptin ELISAS, and Dr. Yuji Ikeno at the Barshop Institute for Longevity and Aging Studies, UTHSCSA, University of Texas, San Antonio for processing the WAT samples for histology.
CHAPTER 5: EFFECTS OF AGE ON WHITE ADIPOSE TISSUE PROTEOMES IN WILD-TYPE MICE

Abstract

With advancing age, the body experiences physiological changes in body composition and WAT distribution, including ectopic accumulation of fat in non-adipose organs, which is probably associated to the development of type 2 diabetes and cardiovascular disease. Ectopic accumulation of fat in aging apparently reflects a decreased expandability of WAT. However, the molecular mechanisms leading to the changes observed in WAT with age are not well understood. Thus, using 2DE followed by MS we compared protein expression profiles of four WAT depots from 12 and 24-month-old WT mice. Body weight, body composition and plasma levels of insulin, leptin and adiponectin (total and HMW) were also determined. Among these variables, only total and HMW adiponectin levels showed differences between age groups, with decreased levels in old age. On the other hand, proteomic results showed increased levels of ENO, pyruvate dehydrogenase E1 β, NAD\(^+\)-dependent isocitrate dehydrogenase α, and ATP synthase subunit β in aged mice, suggesting increased aerobic glucose oxidation in these animals compared to the 12-month-old group. Together with decreased CA-III, these changes in WAT are consistent with impaired insulin sensitivity in advanced age. Also, Cu/Zn superoxide dismutase and two chaperones were increased in WAT depots from aged mice, indicating higher cell stress in aging WAT. The effects of age observed in this study were more prevalent than depot-specific differences. In conclusion, the protein expression changes observed in aging WAT were similar in all the depots and are
consistent with decreased insulin sensitivity and increased oxidative damage in old age. Furthermore, the proteins identified in this study could represent diagnostic or therapeutic targets of age-dependent insulin resistance and oxidative damage in WAT.

Introduction

As reviewed in Chapter 1, age is a main factor affecting body composition and WAT distribution in the body (Berryman et al., 2010; Cartwright et al., 2010; Slawik & Vidal-Puig, 2006). With age, the expandability of WAT is affected, and fat starts to accumulate ectopically in other organs, such as liver and muscle (Slawik & Vidal-Puig, 2006). The toxic effects of fat accumulation in these organs have been related to the increased incidence of type 2 diabetes, dyslipidemia and cardiovascular disease in old age (Slawik & Vidal-Puig, 2006). Lipotoxicity includes the increased generation of ROS, which is a known characteristic of aging (reviewed by Muller et al., 2007). In addition, decreased insulin sensitivity is generally associated with advanced age (DeFronzo, 1981; Narimiya et al., 1984).

Studies on the histology of WAT in guinea pigs have shown that adipocyte size decreases and adipocyte number increases in old age (Pond et al., 1986). This is consistent with the mentioned limited expandability of WAT in aging and with decreased lipogenic actions of insulin. However, the molecular mechanisms behind these changes in WAT are not well understood. For that reason, using 2DE followed by MS, we resolved the protein expression profiles of WAT in 12-month-old (adult) and 24-month-old (aged) WT mice and analyzed their differences. Because age has been suggested to affect WAT
gene expression in a depot-specific manner (Cartwright et al., 2010; Kirkland et al., 1990), we included four WAT depots in this analysis (inguinal, retroperitoneal, mesenteric, and epididymal). Body weight, body composition and plasma levels of insulin, leptin, total and HMW adiponectin were also measured. As expected, our results showed substantial effects of age on WAT protein expression. The protein changes detected are consistent with a decrease in insulin sensitivity and increased ROS production in WAT depots of the aged mice. Further research will determine if any of these proteins could be useful diagnostic markers or therapeutic targets of aging-related insulin resistance and oxidative damage.

Materials and Methods

Mice

Male WT C57BL/6J mice (12 and 24 months old) were used in this study. The 12-month-old group has been described in Chapter 4. Mice were kept on a 12-hour light/dark cycle with food and water provided ad libitum. Procedures were approved by the Ohio University Animal Care and Utilization Committee.

Body weight and body composition

Body weight and body composition were measured within the week prior to sacrifice. Body composition was detected using a quantitative NMR apparatus (Minispec, Bruker Optics, Billerica, MA); two measurements were performed and the average recorded.
Plasma insulin, leptin and adiponectin

Within the two weeks prior to sacrifice, 12 and 24-month-old mice were fasted for eight hours and blood from the tail vein was collected in heparinized tubes. As described in Chapter 4, plasma was then separated by centrifugation at 7,000 g for 10 min and stored at -80 °C until processing. Insulin levels were measured using an ELISA kit from ALPCO Diagnostics, Salem, NH (80-INSFSU-E01). Leptin levels were quantified using an ELISA kit from R&D Systems, Inc., Minneapolis, MN (MOB00). HMW and total adiponectin levels were measured using an ELISA kit from ALPCO Diagnostics (47-ADPMS-E01).

WAT depot samples

Dissections were carried out between 8:00 am and 12:00 pm to avoid variability in protein expression due to circadian rhythms (see Chapter 1). Mice were sacrificed by cervical dislocation and inguinal, retroperitoneal, mesenteric and epididymal WAT were collected and weighed. Samples from six 12-month-old and six 24-month-old mice were snap-frozen in liquid nitrogen and stored at -80°C until processing.

Proteomic analysis

The procedures for 2DE have been described in detail in Chapter 4 and were based on those described previously (Kopchick et al., 2002; List et al., 2007b; Okada et al., 2010; Qiu et al., 2005; Sackmann-Sala et al., 2009) and the results obtained in the
protocol optimization tests described in Chapter 3. For the sake of brevity, they are not described in this Chapter.

**Mass spectrometry**

Protein spots displaying significant intensity changes among age groups and WAT depots were manually excised from the gels and sent to Protea Biosciences Inc., Morgantown, WV for analysis by MS and MSMS using MALDI-TOF and MALDI-TOF-TOF, respectively. These procedures have been described in detail in Chapter 4 and for the sake of brevity will not be listed here. During this study Protea Biosciences updated their data processing software to ProteinPilot 3.0. Thus, some of the data were processed using Applied Biosystems GPS Explorer v3.6 and some using ProteinPilot 3.0.

**Protein identification (Performed at Ohio University)**

Protein identities obtained by Protea Biosciences were verified or revised using the MS and MSMS data obtained and the online software Mascot (www.matrixscience.com). Search parameters were the same as described in Chapter 4.

**Depot-specific protein content**

Protein concentration data measured in the samples treated for proteomics (see Chapter 4 for detailed procedures) were used to estimate the protein content in each of the depots. As described in Chapter 4, the calculation consisted of multiplying the volume of protein solution obtained (in milliliters) by the concentration of isolated
proteins (in milligrams per milliliter) to obtain the total weight of protein isolated (in milligrams). This value was then divided by the initial weight of the processed sample (in grams). The results are expressed in milligrams of protein per gram of tissue.

Statistical analysis

Data for body weight, body composition and plasma hormone levels were compared among age groups using a two-tailed independent-samples $t$-test. The homogeneity of variances between groups was tested with Levene’s test and when homoscedasticity was not met ($P<0.05$), $t$-test results were adjusted.

Spot intensity data were log-transformed. WAT depot weights, protein contents, and log-transformed spot intensities were compared among age groups and depots using a two-way ANOVA with one within-subjects factor (depot) and one between-subjects factor (age). Sphericity was tested using Mauchly’s method, and a Greenhouse-Geisser correction was applied when the assumption of sphericity was not met ($P<0.05$). When significant differences were found among depots and/or age groups, Tukey’s HSD post-hoc tests were performed. Correlations were evaluated using the Pearson test.

Statistical significance cutoffs were $P<0.01$ for spot intensity differences between age groups and among depots (given the intrinsic variability of the 2DE technique) and $P<0.05$ for comparisons of body weight, body composition, plasma hormone levels, WAT depot weights, protein concentrations, and for correlations. The softwares used were SPSS v14.0 and SigmaPlot v11; post-hoc tests for non-spherical data were performed manually in Excel spreadsheets.
Results

Body weight, body composition, and plasma hormone levels

The characteristics of the 12-month-old WT mice (described in Chapter 4) were compared to 24-month-old WT mice. Tables 11 and 12 show mean values for body weight, body composition and plasma hormone levels of the two groups. Although body weight remained very similar between groups, fat mass in absolute and percent values showed a trend to decrease and lean mass a trend to increase as a function of age. Plasma insulin and leptin levels were lower in the aged group, but this difference did not reach significance. The only significant differences that were observed between the groups were for total and HMW adiponectin levels, which were lower in the aged mice. The ratio of HMW to total adiponectin, however, was not significantly different in adult and aged mice. In addition, as seen among 12-month-old mice (Chapter 4), there was a significant correlation between plasma levels of total and HMW adiponectin in the combined groups ($r^2=0.90$, $n=16$, $P<0.001$, Figure 28).
### Table 11

**Body Weight and Body Composition of 12 and 24-Month-Old WT Mice**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Body weight (g)</th>
<th>Fat mass (g)</th>
<th>Fat mass (%)</th>
<th>Lean mass (g)</th>
<th>Lean mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (n=10)</td>
<td>38.8 ± 1.0</td>
<td>9.9 ± 0.8</td>
<td>25.3 ± 1.6</td>
<td>24.9 ± 0.5</td>
<td>64.5 ± 1.2</td>
</tr>
<tr>
<td>24 (n=6)</td>
<td>41.1 ± 3.2</td>
<td>8.1 ± 2.9</td>
<td>17.8 ± 5.3</td>
<td>28.3 ± 1.0</td>
<td>70.4 ± 4.6</td>
</tr>
</tbody>
</table>

All mice were male. Values are mean ± SE. No significant differences were found between age groups on a two-tailed independent-samples t-test.

### Table 12

**Hormone Levels of 12 and 24-Month Old WT Mice**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Total Adip (µg/ml)</th>
<th>HMW Adip (µg/ml)</th>
<th>HMW/Total Adip</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (n=10)</td>
<td>3.16 ± 0.58</td>
<td>31.4 ± 5.1</td>
<td>29.1 ± 2.0*</td>
<td>10.6 ± 1.4*</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>24 (n=6)</td>
<td>1.98 ± 0.55</td>
<td>22.9 ± 14.2</td>
<td>18.5 ± 2.5</td>
<td>5.4 ± 1.2</td>
<td>0.28 ± 0.03</td>
</tr>
</tbody>
</table>

All mice were male. Values are mean ± SE. (*) Significant difference between age groups on a two-tailed independent-samples t-test (total adiponectin, \(P=0.006\); HMW adiponectin, \(P=0.024\)). Abbreviations: Adip, adiponectin.
Figure 28. Correlation between HMW and total adiponectin plasma levels of 12 and 24-month-old WT mice. ○ 12-month-old mice; ■ 24-month-old mice.

Depot-specific weight and protein content

WAT from four depots was weighed at dissection. At the time point selected near the end of their lifespan (24 months of age), we observed that most of the mice (five out of six) had large tumors in the gastrointestinal area (one each). Among these five mice, three also had raw skin on the back of the neck. Interestingly, the aged mouse with no signs of neoplasia or skin problems had the largest WAT depots, body weight and fat mass of the group. Also, insulin and leptin levels in this mouse were outliers among the levels measured for the aged group. This could indicate that this mouse had not experienced the loss of body fat (wasting) characteristic of the end of life and that overall it was healthier than the remaining mice in that group.

As observed in the adult mice, when WAT depot weights for both 12 and 24-month-old mice were analyzed, there were significant differences among depots


Post-hoc pairwise comparisons of the mean depot weights showed that the epididymal depot was significantly larger than the others, and that the inguinal depot was significantly larger than the retroperitoneal pad. Regarding protein content per gram of tissue, the retroperitoneal depot showed a striking increase in protein content with age, and thus, the overall protein content in this depot (and the mesenteric depot) displayed significantly higher levels than inguinal and epididymal WAT ($P<0.001$, Figure 29B).

**Figure 29.** Depot-specific weight and protein content of 12 and 24-month-old WT mice. Data shown are mean ± SE for six male mice except for 12-month-old WAT depot weight means, which include data from 12 mice (○ 12-month-old mice; ■ 24-month-old mice). A two-way ANOVA showed a significant effect of depot ($P<0.001$) with no effect of age or age × depot interaction for depot weight (A), and a significant effect of depot ($P<0.001$) and significant age × depot interaction ($P=0.011$) with no effect of age for protein content (B).

**Correlations of hormone levels to total and depot-specific fat mass**

We evaluated correlations between each of the measured hormones (insulin, leptin, total and HMW adiponectin) and total and depot-specific fat masses. As observed
for the 12-month-old mice (Chapter 4), leptin levels in adult and aged mice correlated positively with total fat mass ($r^2=0.90$, $n=16$, $P<0.001$) (Figure 30). Insulin and total adiponectin levels correlated with fat mass as well, although not as closely as leptin (insulin: $r^2=0.30$, $n=16$, $P=0.027$; total adiponectin: $r^2=0.31$, $n=16$, $P=0.024$) (Figure 31). Despite the tight correlation between total and HMW adiponectin, the latter did not correlate significantly with fat mass ($r^2=0.18$, $n=16$, $P=0.100$). Interestingly, these correlations were much tighter in aged mice when analyzed separately. In fact, in 24-month-old mice, apart from leptin levels, fat mass correlated significantly with insulin ($r^2=0.77$, $n=6$, $P=0.021$), total adiponectin ($r^2=0.95$, $n=6$, $P<0.001$) and HMW adiponectin ($r^2=0.93$, $n=6$, $P=0.002$) (Figure 31).

![Figure 30. Correlations between plasma leptin levels and fat mass in 12 and 24-month-old WT mice (○ 12-month-old mice; ■ 24-month-old mice).](image)
In addition, there were significant positive correlations among insulin, leptin, total and HMW adiponectin levels, even after controlling for fat mass in the combined aged groups (Table 13). Furthermore, given that fat mass correlated significantly and positively with each of the WAT depot weights (inguinal: $r^2=0.86$, $n=18$, $P<0.001$; retroperitoneal: $r^2=0.82$, $n=18$, $P<0.001$; mesenteric: $r^2=0.61$, $n=18$, $P<0.001$;
epididymal: $r^2=0.74$, $n=18$, $P<0.001$), most hormone levels also correlated positively with the weights of the depots (Table 13). However, after controlling for fat mass, the significant correlations only included levels of insulin with leptin ($r^2=0.36$, $df=13$, $P=0.018$), total ($r^2=0.27$, $df=13$, $P=0.047$) and HMW adiponectin levels ($r^2=0.34$, $df=13$, $P=0.021$). Plasma insulin also correlated with mesenteric depot weight ($r^2=0.30$, $df=13$, $P=0.033$), and plasma adiponectin (total and HMW) showed a negative correlation to inguinal depot weight ($r^2=0.40$, $df=13$, $P=0.012$; $r^2=0.35$, $df=13$, $P=0.019$ respectively). All these correlations had values of $r^2<0.50$.

Given the distinctions observed between the two studied age groups in the relationships between hormone levels and body fat mass (Figure 31), we evaluated the correlations between WAT depot weights and hormone levels in the 24-month-old group alone. It is possible that because all circulating hormone levels correlated significantly with fat mass (Figures 30 and 31), and individual WAT depot weights correlated with fat mass as well (inguinal, $r^2=0.98$, $n=6$, $P<0.001$; retroperitoneal, $r^2=0.97$, $n=6$, $P<0.001$; mesenteric, $r^2=0.87$, $n=6$, $P=0.006$; epididymal, $r^2=0.80$, $n=6$, $P=0.016$), we found significant correlations between the four depot weights and all hormone levels measured in the 24-month-old mice (data not shown). However, after controlling for body fat mass, these mice only showed a positive correlation between insulin levels and epididymal WAT depot weight ($r^2=0.92$, $df=3$, $P=0.009$), and a negative correlation between total adiponectin levels and mesenteric WAT depot weight ($r^2=0.78$, $df=3$, $P=0.045$). As seen for the other correlations evaluated in the aged mice, these correlations displayed high $r^2$ values.
### Table 13

*Correlations among Hormone Levels and between Those and Specific WAT Depot Weights in 12 and 24-Month-Old WT Mice*

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Leptin</th>
<th>Total adiponectin</th>
<th>HMW adiponectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma hormone levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>$r^2=0.47; P=0.004^*$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total adiponectin</td>
<td>$r^2=0.45; P=0.005^*$</td>
<td>$r^2=0.39; P=0.010$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMW adiponectin</td>
<td>$r^2=0.46; P=0.004^*$</td>
<td>$r^2=0.29; P=0.030$</td>
<td>$r^2=0.90; P&lt;0.001^*$</td>
<td></td>
</tr>
<tr>
<td><strong>WAT depot weights</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inguinal</td>
<td>$r^2=0.25; P=0.047$</td>
<td>$r^2=0.74; P&lt;0.001$</td>
<td>$r^2=0.10; P=0.223^†$</td>
<td>$r^2=0.04; P=0.475^†$</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>$r^2=0.24; P=0.056$</td>
<td>$r^2=0.67; P&lt;0.001$</td>
<td>$r^2=0.29; P=0.030$</td>
<td>$r^2=0.19; P=0.095$</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>$r^2=0.51; P=0.002^*$</td>
<td>$r^2=0.65; P&lt;0.001$</td>
<td>$r^2=0.46; P=0.004$</td>
<td>$r^2=0.38; P=0.011$</td>
</tr>
<tr>
<td>Epididymal</td>
<td>$r^2=0.37; P=0.013$</td>
<td>$r^2=0.73; P&lt;0.001$</td>
<td>$r^2=0.27; P=0.038$</td>
<td>$r^2=0.16; P=0.127$</td>
</tr>
</tbody>
</table>

Correlations were evaluated using the Pearson test ($n=16$). Significant correlations ($P<0.05$) are shown in bold. All correlations listed were positive. (*Correlations that were significant after controlling for fat mass ($df=13$). (†) The correlations observed between adiponectin (total and HMW) levels and inguinal weight after controlling for fat mass were negative.)
Effects of age on WAT proteomes

WAT samples from six 24-month-old mice were resolved by 2DE and compared to the samples from 12-month-old mice analyzed in Chapter 4. A total of 169 spots were matched among all gels and their intensities evaluated. Twenty spots showed significant effects of age, 17 showed significant depot-specific differences and four displayed significant interactions of age × depot (Figures 32 to 38). Most of the spots that showed significant depot effects had been previously found to vary among depots in 12-month-old mice (Chapter 4). The remaining depot-specific effects also corresponded to protein identities described in Chapter 4, but to different isoforms of those proteins. For example, new isoforms of MYL9 (spot 11b) and CA-III (spot 7f) showed intensity changes among depots in the combined age groups. Protein spot labels for spots mentioned in Chapter 4 were kept the same in this Chapter if the spot also showed significant variations in intensity in this study (e.g. spots 10a, 10b and 10c (ApoA-I) from Chapter 4 were labeled 10a, 10b and 10c in this Chapter). New isoforms of protein identities previously found in Chapter 4 were labeled with the same number but different letters (e.g. two new isoforms of ApoA-I were labeled 10d and 10e).
Figure 32. Representative 2D-gel of WAT. A. Raw image. B. Same gel image showing 39 spots that displayed significant intensity differences ($P<0.01$) among depots (x), between age groups (o), or a significant interaction of age $\times$ depot (+). The dotted square delineates the gel area shown in Figure 33. Numbered labels correspond to protein identities shown in Table 14.
Figure 33. Topographical representation of the intensity of an individual spot (10d) in different WAT depots of a 12-month-old and a 24-month-old mouse. Images were obtained using the 3D-viewer tool of PDQuest Advanced v8.0 (Bio-Rad) after normalization to total density in each gel. The gel area shown corresponds to the dotted square delineated in Figure 32. Spot 10d was selected as a representative spot that displays intensity variations among WAT depots and among age groups.
Figure 34. Log intensity of protein spots that increased significantly with age in WT mice (P<0.01 in a two-way ANOVA with depot as a within-subjects factor and age as a between-subjects factor, n=6 mice per group). Spot labels correspond to gel locations shown in Figure 32. Protein identities were determined by MS and MSMS (Table 14).
Abbreviations: mo, months of age; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal; ApoA-IV, apolipoprotein A-IV; ApoA-I, apolipoprotein A-I; ENO, enolase; ATP5b, ATP synthase subunit β; VIM, vimentin; PDH-E1-B, pyruvate dehydrogenase E1-β; Idh3α, isocitrate [NAD⁺] dehydrogenase α; ERp29, endoplasmic reticulum resident protein 29; Sod1, Cu/Zn superoxide dismutase; PPlase, peptidyl-prolyl cis-trans isomerase; TTR, transthyretin; E-FABP, fatty acid binding protein, epidermal.

Figure 35. Log intensity of protein spots that decreased significantly with age in WT mice (P<0.01 in a two-way ANOVA with depot as a within-subjects factor and age as a between-subjects factor, n=6 mice per group). Spot labels correspond to gel locations shown in Figure 32. Protein identities were determined by MS and MSMS (Table 14). Abbreviations: mo, months of age; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal; CA-III, carbonic anhydrase 3; Alb, albumin; Hb-β1, hemoglobin β-1.
Figure 36. Log intensity of protein spots that displayed a significant effect of depot in 12 and 24-month-old WT mice (P<0.01 in a two-way ANOVA with depot as a within-subjects factor and age as a between-subjects factor, n=6 mice per group). † indicates significant difference in Tukey’s HSD post-hoc test. Spot labels correspond to gel locations shown in Figure 32. Protein identities were determined by MS and MSMS
(Table 14), 5 = actin; 7 = carbonic anhydrase 3; 10 = apolipoprotein A-I; 15 = hemoglobin β-1; 23 = unknown. Abbreviations: mo, months of age; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.

**Figure 37.** Log intensity of protein spots that were present only in the mesenteric depot in 12 and 24-month-old WT mice (n=6 mice per group). Spot labels correspond to gel locations shown in Figure 32. Protein identities are shown in Table 14. Abbreviations: mo, months of age; Mes, mesenteric.
Figure 38. Log intensity of protein spots that displayed a significant interaction of depot \times age in 12 and 24-month-old WT mice (P<0.01 in a two-way ANOVA with depot as a within-subjects factor and age as a between-subjects factor, \( n = 6 \) mice per group). † indicates significant difference in Tukey’s HSD post-hoc test. Spot labels correspond to gel locations shown in Figure 32. Protein identities were determined by MS and MSMS (Table 14): 5 = actin; 10 = apolipoprotein A-I; 24 = EH-domain containing protein 2. Abbreviations: mo, months of age; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.

Using MS and MSMS, 17 unique proteins were identified in the 39 spots that displayed significant intensity changes, with many spots representing isoforms of the same protein (Table 14). As stated in Chapter 4, these isoforms probably result from post-
translational modifications, which generate subtle changes in the pI and/or molecular weight of the proteins. Three spots (17, 22, and 23) could not be identified, mostly because of the low protein amount present in these spots. Table 15 summarizes the proteins identified, how they were affected by age, depot or interaction of age × depot, and their functions (generation of ATP, glucose and lipid metabolism, lipid transport, stress resistance, cytoskeleton structure, etc.).
Table 14

Protein Identities of Spots Displaying Significant Intensity Differences in 12 and 24-Month-Old WT Mice, Determined by MS and MSMS

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<th>Sequence coverage (%)</th>
<th>Score or CI %</th>
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<th>MSMS (up to 3 top scoring peptides)</th>
<th>Error (Da)</th>
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* CI %: Confidence Interval %
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<td>P17742</td>
<td>Peptidyl-prolyl cis-trans isomerase A (PPIase A, cyclophilin A)</td>
<td>6</td>
<td>60</td>
<td>58/53</td>
<td>1</td>
<td>R.IIPGFMCGGDFTR.H</td>
<td>-0.3332</td>
<td>1598.412</td>
<td>17/53</td>
</tr>
<tr>
<td>27</td>
<td>P07309</td>
<td>Transthyretin (TTR, prealbumin)</td>
<td>7</td>
<td>60</td>
<td>77/64</td>
<td>2</td>
<td>K.TLGISPFHEFADVVF.T K.TAESGELHGLTTDEKF VEGVYR.V</td>
<td>-0.3839</td>
<td>2516.832</td>
<td>25/34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 14: continued

<table>
<thead>
<tr>
<th>Spot</th>
<th>Accession number</th>
<th>Protein name, epidermal (E-FABP, fatty acid-binding protein 5)</th>
<th>Unique peptides detected</th>
<th>Sequence coverage (%)</th>
<th>Score or CI %*</th>
<th>Peptides detected by MSMS (up to 3 top scoring peptides)</th>
<th>Error (Da)</th>
<th>m/z</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>Q05816</td>
<td>Fatty acid-binding protein, epidermal</td>
<td>8</td>
<td>42 74/64</td>
<td>4</td>
<td>K.TTVFSCNLGGEKFDETT</td>
<td>-0.2284</td>
<td>2247.790</td>
<td>50/35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ADGR.K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K.ELGVGLALR.K</td>
<td>-0.0762</td>
<td>927.486</td>
<td>39/35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K.TTVFSCNLGGEK.F</td>
<td>-0.1080</td>
<td>1255.491</td>
<td>37/35</td>
</tr>
</tbody>
</table>

Although the NCBInr database was searched, accession numbers listed are from the SWISS-Prot database, so as to unify in an individual entry all the possible matches representing sequence variants of the same protein. (*) Spots identified via manual online searches on Mascot or via ProteinPilot display MS scores; spots identified using the GPS Explorer show confidence intervals (CI %). Peptide mass fingerprint (MS) scores and peptide MSMS scores obtained from manual searches on Mascot are shown as [score/significance cutoff]; confidence intervals (CI%) above 95% are significant (P<0.05). Significant scores and CI% are shown in bold. See materials and methods for a detailed description of the methodology used. (**) For most of the spots labeled “5” only the best scoring isoforms of actin are reported; however, more isoforms were matched with slightly lower scores. (***) The molecular weight of spot 24 and the MSMS fragments matched indicated that the spot contained a C-terminal fragment of EH domain-containing protein 2, however, MS matches included hits to peptides elsewhere in the protein (non-specific hits). MS peptides matching only the C-terminal region of the protein were five, with a total coverage of 13%. No MS matches were obtained using only those peptides,
probably due to low coverage of the protein. This is a common problem encountered when searching for small protein fragments. (†) Spots 5l, 7e and 16 were each identified as mixtures of two different proteins.

Table 15

Summary of Protein Identities, Effects of Age, Depot and Interaction of Age × Depot, and Protein Function for Spots Displaying Significant Intensity Differences in 12 and 24-Month-Old WT Mice (P<0.01)

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Significant effects</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP generation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td>ATP synthase subunit β, mitochondrial</td>
<td>12&lt;24</td>
<td>ATP synthesis</td>
</tr>
<tr>
<td>Glucose/lipid metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6b-c</td>
<td>Enolase</td>
<td>12&lt;24</td>
<td>Glycolysis/glyceroneogenesis</td>
</tr>
<tr>
<td>19</td>
<td>Pyruvate dehydrogenase E1, mitochondrial (PDHE1-B)</td>
<td>12&lt;24</td>
<td>Conversion of pyruvate to acetyl CoA</td>
</tr>
<tr>
<td>Spot</td>
<td>Protein identity</td>
<td>Significant effects</td>
<td>Function</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>---------------------</td>
<td>----------</td>
</tr>
<tr>
<td>20</td>
<td>Isocitrate dehydrogenase</td>
<td>12&lt;24</td>
<td>Krebs cycle</td>
</tr>
<tr>
<td></td>
<td>[NAD$^+$] $\alpha$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7a,d-f*</td>
<td>Carbonic anhydrase 3 (CA-III)</td>
<td>12&gt;24 (d,e)</td>
<td>E&gt;I (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E&gt;M (f)</td>
</tr>
<tr>
<td>28</td>
<td>Fatty acid-binding protein, epidermal (E-FABP)</td>
<td>12&lt;24</td>
<td>Lipolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipid transport</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10a-e</td>
<td>Apolipoprotein A-I (ApoA-I)</td>
<td>12&lt;24 (a,d)</td>
<td>R&gt;E (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I,E: 12&lt;24 (b,c,)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R&gt;I (e)</td>
</tr>
</tbody>
</table>

Table 15: continued
Table 15: continued

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Significant effects</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>51*</td>
<td>Apolipoprotein A-IV (ApoA-IV)</td>
<td>12&lt;24</td>
<td>Cholesterol transport in HDL and chylomicrons, anti-inflammatory functions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stress resistance</td>
</tr>
<tr>
<td>25</td>
<td>Superoxide dismutase [Cu-Zn]</td>
<td>12&lt;24</td>
<td>Anti-oxidant protein</td>
</tr>
<tr>
<td>21</td>
<td>Endoplasmic reticulum resident protein 29 (ERp29)</td>
<td>12&lt;24</td>
<td>Chaperone</td>
</tr>
<tr>
<td>26</td>
<td>Peptidyl-prolyl cis-trans isomerase A (PPIase A, cyclophilin A)</td>
<td>12&lt;24</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Spot</td>
<td>Protein identity</td>
<td>Significant effects</td>
<td>Function</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>5e-l*</td>
<td>Actin (various forms)</td>
<td>12&lt;24 (I)</td>
<td>Microfilaments; muscle contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&gt;I (e,i,j)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&gt;R (e-i)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&gt;E (e,h-k)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>I&gt;E (i)</td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td>Vimentin</td>
<td>12&lt;24</td>
<td>Class-III intermediate filaments</td>
</tr>
<tr>
<td>11a-b</td>
<td>Myosin regulatory light polypeptide 9 (MYL9, myosin regulatory light chain 2, smooth muscle isoform)</td>
<td>Only in M</td>
<td>Smooth muscle contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 15: continued

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Significant effects</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Transgelin (smooth muscle protein 22-α)</td>
<td>Only in M</td>
<td>Actin cross-linking protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>EH domain-containing protein 2</td>
<td>24: I&gt;E</td>
<td>Regulation of endocytosis</td>
</tr>
<tr>
<td>7e</td>
<td>Serum albumin</td>
<td>12&gt;24</td>
<td>Most abundant plasma protein,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>transporter, regulator of colloidal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>osmotic pressure in blood</td>
</tr>
<tr>
<td>15a-d</td>
<td>Hemoglobin subunit β-1</td>
<td>12&gt;24 (c-d)</td>
<td>Oxygen transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&gt;I (a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&gt;E (b)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Annexin A5</td>
<td>12&gt;24</td>
<td>Anticoagulant protein</td>
</tr>
<tr>
<td>Spot</td>
<td>Protein identity</td>
<td>Significant effects</td>
<td>Function</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------------</td>
<td>---------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>27</td>
<td>Transthyretin (prealbumin)</td>
<td>12&lt;24</td>
<td>Binds thyroid hormones and RBP4</td>
</tr>
</tbody>
</table>

Protein identities were obtained by MS and MSMS (Table 14). Spot labels correspond to the gel locations shown in Figure 32. When effects were specific to individual isoforms, the affected isoforms are indicated between parentheses. (*) Spots 5l, 7e and 16 contain a mixture of two proteins, thus they were each listed under two protein identities (however, the intensity changes observed could be due to variation in both or only one of the proteins in the mixture). Spot intensity changes with age, depot and interaction of age × depot are shown in Figures 34-38. Abbreviations: 12, 12 months old; 24, 24 months old; I, inguinal; R, retroperitoneal; M, mesenteric; E, epididymal; RBP4, retinol binding protein 4.
Correlations between spot intensity and circulating hormone levels

Isoform- and depot-specific correlations with plasma hormone levels were found for 30 of the 39 spots that displayed significant intensity changes. Table 16 lists the correlations that explained at least 50% of the variability in the data ($r^2 \geq 0.50$). Consistent with the tight correlation observed between total and HMW adiponectin, many of the spots that correlated with total adiponectin, also showed significant correlations with HMW adiponectin. On the other hand, even though there were significant correlations among all hormone levels, there were hardly any overlaps between the spots that correlated somewhat tightly ($r^2 \geq 0.50$) to insulin or leptin and those that showed similar correlations to total and HMW adiponectin.
### Table 16

**Significant Correlations between Circulating Hormone Levels and Spot Intensities in 12 and 24-Month-Old WT Mice**

<table>
<thead>
<tr>
<th>Protein Spot Depot Correlation</th>
<th>Protein Spot Depot Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
</tr>
<tr>
<td>ApoA-I 10c Ret $r^2=0.79; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td>ApoA-I 10d Ret $r^2=0.50; P=0.010$</td>
<td></td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
</tr>
<tr>
<td>Hb-β1 15a Epi $r^2=0.63; P=0.002$</td>
<td>PDHE1-B 19 Epi $r^2=0.51; P=0.009$</td>
</tr>
<tr>
<td>Hb-β1 15b Epi $r^2=0.67; P=0.001$</td>
<td>Actin 5j Epi $r^2=0.60; P=0.003$</td>
</tr>
<tr>
<td>Unknown 17 Epi $r^2=0.56; P=0.005$</td>
<td></td>
</tr>
<tr>
<td><strong>Total adiponectin</strong></td>
<td></td>
</tr>
<tr>
<td>CA-III 7d Ing $r^2=0.74; P&lt;0.001$</td>
<td>Enolase 6b Epi $r^2=0.61; P=0.003$</td>
</tr>
<tr>
<td>CA-III 7f Ret $r^2=0.55; P=0.006$</td>
<td>Enolase 6c Ret $r^2=0.62; P=0.002$</td>
</tr>
<tr>
<td>Actin 5i Ing $r^2=0.51; P=0.009$</td>
<td>Idh3α 20 Epi $r^2=0.55; P=0.006$</td>
</tr>
</tbody>
</table>
Table 16: continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-Kβ1</td>
<td>15d</td>
<td>Ing</td>
<td>$r^2=0.51$; $P=0.009$</td>
<td>E-FABP</td>
<td>28</td>
<td>Epi</td>
<td>$r^2=0.61$; $P=0.003$</td>
</tr>
<tr>
<td>AnnexinA5</td>
<td>18</td>
<td>Ret</td>
<td>$r^2=0.71$; $P=0.001$</td>
<td>Actin/ApoA-IV</td>
<td>51</td>
<td>Ret</td>
<td>$r^2=0.52$; $P=0.008$</td>
</tr>
<tr>
<td>Unknown</td>
<td>22</td>
<td>Ret</td>
<td>$r^2=0.57$; $P=0.005$</td>
<td>PPIase</td>
<td>26</td>
<td>Ret</td>
<td>$r^2=0.53$; $P=0.007$</td>
</tr>
<tr>
<td>Unknown</td>
<td>17</td>
<td>Epi</td>
<td>$r^2=0.61$; $P=0.003$</td>
<td>CAKIII</td>
<td>7d</td>
<td>Ing</td>
<td>$r^2=0.62$; $P=0.002$</td>
</tr>
<tr>
<td>CA-III</td>
<td>7f</td>
<td>Ret</td>
<td>$r^2=0.56$; $P=0.005$</td>
<td>ApoA-I</td>
<td>10e</td>
<td>Ret</td>
<td>$r^2=0.52$; $P=0.008$</td>
</tr>
<tr>
<td>AnnexinA5</td>
<td>18</td>
<td>Ret</td>
<td>$r^2=0.61$; $P=0.003$</td>
<td>PPIase</td>
<td>26</td>
<td>Ret</td>
<td>$r^2=0.54$; $P=0.007$</td>
</tr>
</tbody>
</table>

HMW adiponectin

Results correspond to $r^2$ and $P$ values for Pearson correlation tests. Abbreviations: ApoA-I, apolipoprotein A-I; Hb-β1, hemoglobin β-1; PDHE1-B, pyruvate dehydrogenase E1 subunit β; CA-III, carbonic anhydrase 3; Idh3α, isocitrate [NAD$^+$] dehydrogenase α; E-FABP, fatty acid binding protein, epidermal; ApoA-IV, apolipoprotein A-IV; PPIase, peptidyl-prolyl cis-trans isomerase; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.
Discussion

The goal of this study was to analyze the effects of age on WAT depot proteomes using 12-month-old (adult) and 24-month-old (aged) WT mice. Body weight, fat mass and lean mass did not show significant changes between age groups, although there were trends for increased lean mass and decreased fat mass in aged animals. Given the advanced age of mice in the 24-month-old group, their fat mass and lean mass were expected to be lower than in the adult group. However, only fat mass tended to decrease in that group. The decrease in lean mass that is usually observed in elderly humans (Miller & Wolfe, 2008) was not present in our aged mice. Berryman et al. (2010) reported similar results, suggesting that lean mass does not decrease in aging mice. At the same time, circulating levels of insulin, leptin, and total and HMW adiponectin were all decreased in 24-month-old mice, but only the decrease in total and HMW adiponectin was statistically significant. Decreased adiponectin action is consistent with the insulin resistant state associated with old age (DeFronzo, 1981; Narimiya et al., 1984). However, the ratio of HMW to total adiponectin was not different between the age groups.

The weights of the four WAT depots analyzed were similar in 12 and 24-month-old mice, although a tendency for lower weight was observed in the aged mice. Depot-specific differences observed in adult animals (Chapter 4) were conserved in the aged group. The epididymal depot was the largest and the retroperitoneal depot the smallest in combined age groups. However, contrary to what was observed in the 12-month-old group, the mesenteric depot was not significantly larger than the retroperitoneal depot. Protein content per gram of tissue tended to increase with age in all depots, and showed a
very large increase in the retroperitoneal depot ($P=0.011$ for age $\times$ depot interaction). As stated previously, aging in WAT depots is characterized by a reduction in cell size but maintained or increased cell numbers (Pond et al., 1986). Increased protein content per gram of tissue might thus be associated to the loss of lipid in the adipocytes, resulting in a relative increase in WAT protein content. The large increase observed in retroperitoneal WAT might reflect a marked decrease in adipocyte size. In fact, at 12 months of age, the retroperitoneal depot had the second-largest mean adipocyte size of the four fat pads. If the size of adipocytes in this depot is largely reduced with age, then that could explain the increased protein content per gram of tissue observed at 24 months of age. Unfortunately, adipocyte sizes were not measured in WAT depots of mice from the aged group. On the other hand, higher circulation or innervation in this depot at old age also could explain the increased protein content, but decreased hemoglobin-β levels suggest no increase in blood flow with age in this depot (see below). Histological analysis of the WAT depots of 24-month-old mice would possibly clarify this matter.

Correlations were tested between fat mass and the four plasma hormone levels measured. Strong positive correlations between fat mass and plasma levels of insulin, and total and HMW adiponectin were observed only in the mice from the aged group. This might be due to the range of fat mass values being wider in this group than that of the 12-month-old mice. Regarding circulating adiponectin, an inverse relationship with body fat mass is usually observed, with obese individuals displaying low adiponectin levels (Kaser et al., 2008). The reason why we observed a positive correlation (rather than a negative one) between adiponectin (total and HMW) and fat mass might be that the mice studied
did not have excessive abundance of body fat. As stated in Chapter 4, it is possible that the negative association between adiposity and adiponectin levels develops only when increases in body fat are drastic or when insulin sensitivity is severely affected, as seen in ob/ob and db/db mice (Hu et al., 1996; Yamauchi et al., 2001). Relative to this, it was interesting to find a negative correlation between mesenteric fat mass and total adiponectin levels in the aged group of mice, which is consistent with the documented influence of visceral fat on adiponectin levels (Cote et al., 2005; Drolet et al., 2009; Kwon et al., 2005) and with the expected decrease in insulin sensitivity at old age (DeFronzo, 1981; Narimiya et al., 1984). Regarding leptin, its levels correlated closely to fat mass in both age groups, as was observed for the 12-month-old mice in Chapter 4. As stated in that Chapter, this is consistent with what is generally observed in humans and mice (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1995). Also, higher body fat mass usually correlates with higher insulin levels even in elderly people (Carantoni et al., 1998), therefore, the positive correlation found between insulin and body fat mass was not surprising.

In addition, contrary to what was observed in 12-month-old mice (Chapter 4), the combined age groups showed significant correlations among insulin, leptin, total and HMW adiponectin levels. Possibly, mice with higher insulin levels respond with enhanced lipogenesis in WAT and higher leptin secretion (Kieffer & Habener, 2000). Adiponectin production might also be induced by insulin, but this concept is still controversial (Hernandez-Morante et al., 2008). On the other hand, the tight positive correlation found in the 24-month-old mice between insulin levels and epididymal fat
mass after controlling for total body fat may indicate an increased response to insulin’s lipogenic and antilipolytic actions in this depot at old age. However, this contradicts the general notion of low insulin sensitivity in intra-abdominal WAT depots (Wajchenberg, 2000). Therefore, further studies are necessary to clarify the source of this correlation.

The protein profiles of WAT depots from 24-month-old WT mice were resolved and compared to those of 12-month-old mice to evaluate the effects of age on WAT protein expression. Interesting age effects on various proteins were found. Most of the spots affected by age showed increased levels in the aged group of mice. These spot identities included proteins involved in ATP generation, glycolysis/glyceroneogenesis, Krebs cycle, and stress resistance, among others.

The ATP synthase subunit β was present in a spot mixed together with vimentin. Unfortunately, establishing which protein in the mixture is responsible for the change in intensity is not possible with the data obtained. However, ATP synthase subunits were also increased in aged versus young adult rat muscle in whole tissue samples (Donoghue et al., 2010) and in mitochondria-enriched fractions (O’Connell & Ohlendieck, 2009). Therefore, increased levels of ATP generation in WAT of aged mice are consistent with what was reported for rat muscle.

Two isoforms of ENO, different from the isoform mentioned in Chapter 4, showed higher intensity in aged than adult mice. As already stated, this enzyme plays a role in both glycolysis and glyceroneogenesis, and the detected changes in expression could reflect increased activity of either pathway. Several tissues, including liver and skeletal muscle, have been reported to display impaired mitochondrial function in aging
humans and various animal models (Petersen et al., 2003; Wei et al., 1998), with a concomitant age-dependent shift towards increased glycolytic activity as the source of ATP (Wei et al., 2009). However, these data were lately contradicted by studies in rats showing increased oxidative metabolism in aging skeletal muscle (Donoghue et al., 2010; Doran et al., 2009; O'Connell & Ohlendieck, 2009). The latter studies report increased levels of ENO and ATP synthase in muscle of old rats, which are consistent with our results in mouse WAT.

Adding support to the hypothesis of increased oxidative metabolism in aging, our data showed increased levels of pyruvate dehydrogenase E1 subunit β (PDHE1-B) and isocitrate dehydrogenase [NAD⁺] α (Idh3α) in WAT of aged mice. PDHE1-B is part of the pyruvate dehydrogenase complex (PDC), which is activated by insulin and catalyzes the irreversible oxidative decarboxylation of pyruvate into acetyl-CoA (Denton et al., 1989; Moule & Denton, 1997). Given that PDHE1-B is regulated by phosphorylation, increased protein levels of this enzyme do not necessarily reflect an increase in enzyme activity. On the other hand, Idh3α is a subunit of Idh3, which participates in the Krebs cycle by converting isocitrate and NAD⁺ into α-ketoglutarate, CO₂ and NADH, an important point of control in the pathway, regulated by Ca²⁺, ADP, and citrate (activators) and by NADH, NADPH and ATP (inhibitors) (Gabriel et al., 1986). Increased levels of this enzyme were also found in aged rat muscle (Donoghue et al., 2010) and splenic lymphocytes of senescence-accelerated mice (Luo et al., 2009), and indicate enhanced activity of the Krebs cycle. High Krebs cycle activity is consistent with high availability of acetyl-CoA and increased production of pyruvate through glycolysis.
Therefore, our results suggest that, compared to 12-month-old mice, WAT in aged mice displays higher rates of glycolysis, conversion of pyruvate to acetyl-CoA and Krebs cycle activity. The increased levels of ATP synthase observed in the aged group (see above), suggest that the oxidation of glucose continues through the electron transport chain and results in enhanced ATP synthesis via oxidative phosphorylation (Figure 39). The fact that in aged mice acetyl-CoA seems to be channeled to the Krebs cycle rather than be utilized as a substrate for de novo fatty acid synthesis is consistent with the decrease in insulin sensitivity associated with old age (DeFronzo, 1981; Narimiya et al., 1984). Considering the insulin-sensitizing actions of adiponectin, the proposed scenario agrees with the negative correlations found between total plasma adiponectin levels and isoforms of ENO and Idh3α and the positive correlations between total and HMW adiponectin and CA-III isoforms (see below).
Figure 39. Schematic summary of the metabolic pathways activated in WAT of 24-month-old WT mice, as indicated by proteomic results. Increased levels of enolase, pyruvate dehydrogenase E1, isocitrate [NAD] dehydrogenase, and ATP synthase suggest an activation of aerobic glucose oxidation, leading to increased production of ATP and of ROS as by-products. In agreement with an increased production of ROS, the levels of three stress-resistant proteins were increased. Also, consistent with the activation of glycolysis and the Krebs cycle, low levels of CA-III suggest decreased glyceroneogenesis and de novo fatty acid synthesis. On the other hand, high E-FABP levels indicate enhanced lipolysis, which probably results in increased release of fatty acids to the bloodstream, given that G3P production through glyceroneogenesis is apparently low. These results were similar in all the WAT depots studied and they are consistent with insulin resistance and high oxidative stress in WAT at old age. However, depot-specific differences were found in the levels of the C-terminal fragment of EH domain-containing protein 2, which were higher in the inguinal than the epididymal depot. Levels of this protein suggest a prolonged presence of GLUT4 in the cell membrane in the inguinal depot (shown in dashed rectangle). Thick and thin arrows symbolize high and low activation respectively of reactions/pathways involved. Proteins that increased are shown in green, and proteins that decreased are shown in red. Abbreviations: GLUT4, glucose transporter 4; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; E-FABP, epidermal fatty acid binding protein; CA-III, carbonic anhydrase 3; ROS, reactive oxygen species; ER, endoplasmic reticulum.
Furthermore, increased levels of epidermal fatty acid binding protein (E-FABP) in WAT from aged mice are consistent with increased lipolysis and decreased lipogenesis (Figure 39), suggesting decreased insulin sensitivity, also consistent with the negative correlations found between E-FABP and adiponectin (total and HMW) levels. E-FABP is the minor lipid chaperone in adipocytes, where adipocyte FABP (A-FABP) represents the majority of the lipid chaperone activity. These two proteins are thought to bear overlapping functions, which mainly involve binding to HSL to facilitate lipolysis and fatty acid efflux from the cell (Hertzel et al., 2002). In fact, lipolytic activity was shown to correlate to total FABP levels but not to a specific type of FABP (Hertzel et al., 2002). Consistent with our results, E-FABP levels were also found to increase in senescent human dermal microvascular endothelial cells in culture (Ha et al., 2004) and in aging rat muscle (Doran et al., 2009). The age-dependent decrease in adipocyte size (Pond et al., 1986) also agrees with increased lipolysis. On the other hand, a possible antioxidant role was proposed for E-FABP in retinal epithelial cells, given its ability to bind 4-hydroxynonenal, a natural product of lipid peroxidation (Bennaars-Eiden et al., 2002).

Because aging is associated with increased oxidative damage, higher levels of E-FABP in old age would also be consistent with its putative antioxidant function.

Proteins with recognized stress-resistant functions were increased at old age, including Cu/Zn superoxide dismutase (Sod1) and two chaperones: peptidyl-prolyl cis-trans isomerase A (PPIase A) and endoplasmic reticulum resident protein 29 (ERp29) (Figure 39). As already stated, increased levels of these proteins are consistent with the increased oxidative stress widely associated with aging (reviewed by Muller et al., 2007).
Elevated levels of antioxidant proteins and chaperones were also detected in mitochondria-enriched fractions of aging rat muscle (O'Connell & Ohlendieck, 2009).

Regarding lipid transport, two isoforms of ApoA-I showed increased levels in WAT of aged mice, suggesting that the rate of cholesterol transport from WAT to the liver increases as animals age. As stated in Chapter 4, HDL particles bind to adipocyte membranes in WAT (Fong et al., 1986), and could reflect blood flow in WAT depots. However, blood flow in the mesenteric depot of old obese rats was found to be lower than in young rats or in old rats subjected to CR, and blood flow in the SC depot was lower in both groups of old animals (CR and obese) than young rats (Crandall et al., 1984). In addition, no differences in WAT blood flow were found between old and young rats in the epididymal and retroperitoneal depots (Crandall et al., 1984). Thus, if any changes in blood flow were to be found when comparing adult and aged mice, decreased rather than increased values would be expected in old age. Therefore, increased ApoA-I levels in aged mice probably do not reflect increased blood flow in WAT depots with age. On the other hand, increased ApoA-I levels might also reflect increased anti-inflammatory action to counteract age-dependent endothelial damage (Barter et al., 2007). As mentioned in Chapter 4, anti-inflammatory functions have been proposed for ApoA-I and HDL (Barter et al., 2007).

In addition, increased intensity of spot 5l, which contained a mixture of ApoA-IV and actin, is consistent with higher HDL levels at old age. In agreement with our data, both ApoA-I and ApoA-IV serum levels correlate with age in humans (Sun et al., 2000), and ApoA-I increases with age in mice (Araki & Goto, 2003). However, levels of ApoA-
IV and HDL were reported to be similar between young and aged mice (Araki & Goto, 2003). Further studies may clarify these inconsistencies.

Finally, one isoform of transthyretin (TTR) showed increased levels in aged mice. This protein is the main transporter of thyroid hormones in rodent plasma, although TTR-null mice have normal thyroid hormone metabolism (Palha et al., 1994). TTR also binds RBP4 in plasma, decreasing its clearance (The UniProt Consortium, 2009). Thus, elevated TTR production in WAT might serve to increase circulating levels of RBP4 in aged mice. Serum RBP4 levels are also increased with age in humans and correlate negatively with insulin sensitivity (Lim et al., 2010; Suh et al., 2010).

Among the proteins that decreased in WAT of aged mice when compared to adult mice were two isoforms of CA-III (one of which was present in a protein mixture with albumin). As already explained, the function of this enzyme in WAT is not completely understood, but a role in the regulation of intracellular pH upon lipolysis and in the supply of bicarbonate for the synthesis of G3P from pyruvate and for de novo synthesis of fatty acids has been proposed (Coulson & Herbert, 1984; Herbert & Coulson, 1984; Herbert et al., 1983). Low CA-III levels could thus be attributed to decreased lipolysis in aged mice, which agrees with human data showing decreased responsiveness to catecholamine stimulation in old age (Arner, 2005; Imbeault et al., 2000; Lonnqvist et al., 1990). However, this interpretation contradicts the increased E-FABP levels measured in our mice (see above). Another explanation for the low CA-III levels detected is that anabolic reactions, such as de novo fatty acid synthesis or glyceroneogenesis, are decreased in WAT of aged mice (Figure 39). This scenario is consistent with the high
PDHE1-B activity detected and with the utilization of acetyl-CoA in the Krebs cycle, as mentioned above. Therefore, low CA-III levels might reflect decreased sensitivity to insulin at old age. In agreement with our data, low CA-III levels were also found in aging rat muscle (Doran et al., 2009).

An anticoagulant protein, annexin A5, was also decreased in WAT of aged mice (especially in retroperitoneal and epididymal depots). The decrease in annexin A5 could be related to the pro-thrombotic state associated with cardiovascular disease, for which advanced age is a known risk factor (Jousilahti et al., 1999). Two isoforms of hemoglobin-β were also decreased in the aged mice, consistent with a possible age-dependent decrease in WAT blood flow as mentioned above. The observed decrease in hemoglobin-β isoforms, however, were more marked in retroperitoneal and epididymal depots, which were the WAT depots that showed no age effects on blood flow in rats (Crandall et al., 1984). Nevertheless, the fact that other isoforms of hemoglobin-β were not affected by age makes it difficult to assess what (if any) changes in blood flow are reflected by the decreased hemoglobin-β isoforms.

Regarding intensity differences among WAT depots, several spots displayed depot-specific patterns that were conserved in age. In fact, isoforms of actin, CA-III, ApoA-I and hemoglobin-β were found to display significant effects of depot in the combined age groups, thus overlapping with the results obtained in Chapter 4. ApoA-I isoforms displayed higher levels in the retroperitoneal depot; actin and hemoglobin-β isoforms were higher in the mesenteric depot; and CA-III isoforms were higher in the epididymal depot, indicating higher lipolysis, de novo fatty acid synthesis or overall lipid
turnover in this depot. Also, MYL9 and transgelin were once again only detected in the mesenteric depot, including one more isoform of MYL9 that displayed very low intensity levels in adult mice and was not detected until the aged group was analyzed. In fact, age changes for this MYL9 isoform yielded a $P$-value of 0.01, but given that the chosen cut-off for significance was $P<0.01$, this spot was not listed among those that show significant age effects. As for the mentioned actin and hemoglobin-β isoforms, and the conserved presence of smooth muscle proteins in mesenteric WAT, these data suggest that, as mice age, blood vessel density in this depot is still high relative to the other fat pads.

Only one isoform of ApoA-I (spot 10d) showed significant effects of the two main factors analyzed (age and depot). For the other proteins mentioned, the intensity of certain isoforms changed with age, while other isoforms were subject to depot-specific changes. This complex isoform-specific regulation would be difficult or even impossible to detect when measuring total protein levels by conventional assays and thus highlights the advantages of 2DE as a tool for investigating protein expression.

Four spots showed intensity changes displaying significant interactions of age × depot. One isoform of actin (spot 5k), which also displayed significant depot effects, showed higher levels in mesenteric than retroperitoneal WAT only in 12-month old mice. The intensity of that spot increased with age in the retroperitoneal depot, therefore causing the difference between the two depots to disappear. Two isoforms of ApoA-I that showed depot effects in the previous Chapter (spots 10b and 10c), displayed significant interactions of age × depot when the aged group was added for this analysis. In 12-
month-old animals, the intensity of these isoforms in inguinal and epididymal WAT was lower than in aged mice. Therefore, the increase observed in inguinal and epididymal WAT for ApoA-I isoforms 10b and 10c parallels the age-dependent increase detected for 10a and 10d in all depots (see above). A fourth spot, identified as a C-terminal fragment of EH domain-containing protein 2 (Ehd2) was higher in intensity in the inguinal depot than the epididymal depot, but only at 24 months of age. This protein has been associated to endocytic processes, including internalization of GLUT4 from the cytoplasmic membrane to endosomes in cultured adipocytes (Guilherme et al., 2004). Interestingly, the C-terminal fragment of Ehd2 contains the EH domain and a short acidic sequence, which have been suggested to mediate binding to F-actin and have a regulatory role in endocytosis (Guilherme et al., 2004). Thus, increased levels of the C-terminal fragment of Ehd2 could indicate that in old age, GLUT4 endocytosis is lower in inguinal WAT than in epididymal WAT, suggesting that in the inguinal depot GLUT4 could remain on the cell membrane for a longer period of time upon insulin stimulation. However, the role of Ehd2 on endocytosis probably affects other membrane transporters and receptors (as seen for transferrin in COS cells), rather than being specific for GLUT4 (Guilherme et al., 2004).

Finally, three spots could not be identified by MS, probably due to the low amounts of these proteins in the gels: spot 17 showed increased intensity with age, spot 22 decreased with age (especially in the retroperitoneal and epididymal depots), and spot 23 showed higher intensity in the epididymal than the retroperitoneal WAT depot. One major problem encountered in this study was that most of the mice in the aged group
showed tumors in the gastrointestinal area and raw skin on the back of the neck. These signs of disease suggest the presence of confounding factors in this analysis, because the pathological status of the mice studied might influence body composition, hormone levels, and protein expression of WAT depots in these mice. However, mesenteric tumors and raw skin are a common finding in aged C57Bl6/J mice (Ward, 2006; and our unpublished observations) and cannot be ruled out as part of their normal aging phenotype.

In summary, when 12 and 24-month-old WT mice were compared, there were no marked differences between age groups regarding body weight, fat mass and lean mass, or circulating hormone levels, except for a decrease in adiponectin levels (total and HMW) observed in the aged group. Interestingly, correlations among hormone levels and between those and individual depot weights were tighter when the aged group of mice was considered alone. Regarding WAT protein expression profiles, several differences were found between age groups. Overall, metabolism in aging WAT appeared to be shifted towards the oxidation of glucose via glycolysis, Krebs cycle and the electron transport chain, with ATP generation via oxidative phosphorylation (Figure 39). Also, fatty acid and TG synthesis seemed to be decreased and stress resistant proteins increased (Figure 39), consistent with the impaired insulin sensitivity and increased oxidative damage usually observed at old age (Figure 40). Interestingly, proteins displaying depot-specific differences in expression showed the same trends that were observed in Chapter 4 and suggested increased blood vessel density in the mesenteric depot and higher TG turnover in the epididymal depot.
Figure 40. Schematic summary of WAT depot characteristics in 12 and 24-month-old WT mice. Arrows indicate the levels (high ↑, low ↓, variable ↔) of metabolic rate and stress in each depot. Sizes of upward arrows (↑) also indicate varying degrees of increased metabolism and stress, where smaller sizes represent lower levels than larger sizes (e.g. at 24 months, levels are high in all depots, however metabolism in inguinal WAT < retroperitoneal and mesenteric WAT < epididymal WAT). These values are based on measured protein levels of metabolic enzymes and stress-resistant proteins. GLUT4 presence on the cell membrane is based on the measured levels of EH domain-containing protein 2. Insulin sensitivity has been estimated in each WAT depot and is represented using a colored scale. Predicted degrees of insulin sensitivity are based on the estimated levels of metabolism and stress, and on the presence of GLUT4 on the membrane. Abbreviations: mo, months; GLUT4, glucose transporter 4.

In conclusion, age-dependent changes in WAT of WT mice are consistent with impaired insulin sensitivity and enhanced oxidative damage in aged mice, showing increased glucose oxidation, decreased lipogenesis and increased levels of stress-resistant proteins. Even though the distinctions between WAT depots described in Chapter 4 were marked, the data obtained in this Chapter show more pronounced effects of age on WAT than effects of depot. In fact, our data suggest that all WAT depots undergo similar
changes in their response to insulin and oxidative stress with advancing age, and that the inguinal depot displays lower metabolic rate than the epididymal depot at both ages (Figure 40). Further research will help establish if the WAT proteins shown to change with age in this study could be used as targets for treatment and/or diagnostic markers of insulin resistance and related aging pathologies in WAT.
CHAPTER 6: WHITE ADIPOSE TISSUE PROTEOMES IN GHR-/- MICE: COMPARISON TO WILD-TYPE DEPOTS AND THEIR AGE-RELATED CHANGES

Abstract

GHR-/- mice are dwarf and display enhanced insulin sensitivity and prolonged longevity in spite of increased fat accumulation. However, only some WAT depots are disproportionately enlarged in GHR-/- mice. Therefore, these mice are a valuable tool to study WAT depot-specific effects on insulin responsiveness and lifespan. Protein profiles of WAT depots from 12 and 24-month-old GHR-/- mice were resolved by 2DE and MS and compared to corresponding profiles of WT mice. Plasma levels of insulin, leptin and adiponectin (total and HMW) were also determined. As expected, insulin levels were lower and adiponectin levels were higher in GHR-/- than WT mice. No differences in leptin levels were detected. The proteomic analysis showed numerous spots to behave similarly in individual WAT depots and age groups of WT and GHR-/- mice. Still, the intensity of twelve spots showed significant effects of genotype or significant interactions of genotype × age, genotype × depot and/or genotype × depot × age. Some of these spots contained proteins involved in membrane protein recycling and senescence-related processes. These protein changes might be responsible for the increased insulin sensitivity and extended lifespan found in GHR-/- mice.

Introduction

As stated in Chapter 1, WAT is an endocrine organ with a myriad of functions, related to the regulation of metabolism, inflammation and immunity, hemostasis, blood
pressure, glucocorticoid and steroid hormone synthesis, and others (Trayhurn, 2005).

Moreover, substantial distinctions have been described for individual WAT depots regarding their contributions to these functions and their cell characteristics (Cartwright et al., 2007; Tran et al., 2008; Wajchenberg et al., 2002).

GHR-/− mice constitute a valuable tool for the study of depot-specific effects on insulin responsiveness and aging. As described in Chapter 1, GHR-/− mice lack GH action due to a disruption of the GHR/BP gene (Zhou et al., 1997). These mice are dwarf, with organ weights reduced proportionally, except for disproportionately decreased liver and kidneys and increased brain and AT (Berryman et al., 2006; Coschigano et al., 2003). Interestingly, despite their high fat mass percentage, GHR-/− mice display high insulin sensitivity and extended longevity (Coschigano et al., 2003). In addition, the accumulation of AT has been shown to localize mainly to the SC region (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Flint et al., 2006; Y. Li et al., 2003; Liu et al., 2004), although increased retroperitoneal WAT also has been reported (Berryman et al., 2004; Egecioglu et al., 2006). Epididymal and mesenteric WAT, on the other hand, generally show no differences in percent depot weight when GHR-/− and control mice are compared (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Egecioglu et al., 2006; Y. Li et al., 2003).

In this study, the already resolved protein profiles of inguinal, retroperitoneal, mesenteric and epididymal WAT depots of 12 and 24-month-old WT mice (Chapters 4 and 5) were compared to corresponding protein profiles in GHR-/− mice. We hypothesized that, compared to WT, GHR-/− mice would display different WAT
proteomes in individual depots depending on the age of the animal and the specific WAT depot under consideration. We found numerous similarities in protein expression profiles of WAT depots between GHR-/- and WT mice. However, proteins involved in cytoskeletal reorganization and senescence-related processes displayed different levels between genotypes. These changes might be responsible for increased insulin sensitivity and extended lifespan in GHR-/- mice.

Materials and Methods

Mice

Male GHR-/- and WT C57BL/6J mice (12 and 24 months old) were used in this study. The 12 and 24-month-old groups of WT mice have been described in Chapters 4 and 5. All mice were kept on a 12-hour light/dark cycle with food and water provided ad libitum. Procedures were approved by the Ohio University Animal Care and Utilization Committee.

Body weight and body composition

These procedures have been described in Chapters 4 and 5. Body weight and body composition were measured within the week prior to sacrifice. Body composition was detected using a quantitative NMR apparatus (Minispec, Bruker Optics, Billerica, MA); two measurements were performed and the average recorded.
Plasma insulin, leptin and adiponectin

As described in Chapter 5, within the two weeks prior to sacrifice, 12 and 24-month-old GHR-/ and WT mice were fasted for eight hours and blood from the tail vein was collected in heparinized tubes. Plasma was then separated by centrifugation at 7,000 g for 10 min and stored at -80 °C until processing. Insulin levels were measured using an ELISA kit from ALPCO Diagnostics, Salem, NH (80-INSMSU-E01). Leptin levels were quantified using an ELISA kit from R&D Systems, Inc., Minneapolis, MN (MOB00). HMW and total adiponectin levels were measured using an ELISA kit from ALPCO Diagnostics (47-ADPMS-E01).

WAT depot samples

GHR-/ mice were sacrificed and their tissues harvested at the same time as the WT mouse groups. Procedures were as described in previous chapters. Dissections were carried out between 8:00 am and 12:00 pm to avoid variability in protein expression due to circadian rhythms (see Chapter 1). Mice were sacrificed by cervical dislocation and inguinal, retroperitoneal, mesenteric and epididymal WAT were collected and weighed. Samples from six 12-month-old and six 24-month-old mice of each genotype were snap-frozen in liquid nitrogen and stored at -80°C until processing.

Proteomic analysis

The procedures for 2DE have been described in detail in Chapter 4 and were based on those described previously (Kopchick et al., 2002; List et al., 2007b; Okada et
al., 2010; Qiu et al., 2005; Sackmann-Sala et al., 2009) and the results obtained in the optimization tests described in Chapter 3. For the sake of brevity, they are not described in this Chapter.

**Mass spectrometry**

Protein spots displaying significant intensity changes between genotypes, age groups or among WAT depots were manually excised from the gels and sent to Protea Biosciences Inc., Morgantown, WV for analysis by MS and MSMS using MALDI-TOF and MALDI-TOF-TOF respectively. These procedures have been described in detail in Chapter 4 and 5 and for the sake of brevity will not be listed here.

**Protein identification (Performed at Ohio University)**

Protein identities obtained by Protea Biosciences were verified or revised using the MS and MSMS data obtained and the online software Mascot (www.matrixscience.com). Search parameters were the same as described in Chapter 4.

**Depot-specific protein content**

Protein concentration data measured in the samples treated for proteomics (see Chapter 4 for detailed procedures) were used to estimate the protein content in each of the depots. As described in Chapters 4 and 5, the calculation consisted of multiplying the volume of protein solution obtained (in milliliters) by the concentration of isolated proteins (in milligrams per milliliter) to obtain the total weight of protein isolated (in
milligrams). This value was then divided by the initial weight of the processed sample (in grams). The results are expressed in milligrams of protein per gram of tissue.

Statistical analysis

Data for body weight, body composition and plasma hormone levels were compared among genotypes and age groups using a two-way ANOVA. Spot intensity data were log-transformed. WAT depot weights, protein contents, and log-transformed spot intensities were compared among genotypes, age groups and depots using a three-way ANOVA with one within-subjects factor (depot) and two between-subjects factors (genotype and age). Sphericity was tested using Mauchly’s method, and a Greenhouse-Geisser correction was applied when the assumption of sphericity was not met ($P<0.05$). Depot weights, protein contents and spot intensities of spots displaying significant interactions of genotype × depot or genotype × depot × age were also evaluated separately in each WAT depot using a two-way ANOVA with age and genotype as between-subjects factors. When significant differences were found between genotypes or age groups, Tukey’s HSD post-hoc tests were performed. Correlations were evaluated using the Pearson test.

Statistical significance cutoffs were $P<0.01$ for spot intensity differences (given the intrinsic variability of the 2DE technique) and $P<0.05$ for comparisons of body weight, body composition, plasma hormone levels, WAT depot weights, protein concentrations, and for correlations. The softwares used were SPSS v14.0 and SigmaPlot v11.
Results

Body weight, body composition, and plasma hormone levels

The characteristics of the 12 and 24-month-old groups of GHR−/− mice were compared to those of the WT mice described in Chapter 5. Mean values for body weight, body composition and plasma hormone levels of the four groups are listed in Table 17, including the main factors (genotype and age) and interaction (genotype × age) that had significant effects on the variables measured. As expected, body weight was lower in the GHR−/− mice, which also displayed higher percent fat mass and lower percent lean mass. Although no changes were observed for body weight with age, a decline in percent fat mass and increased percent lean mass were detected when 24-month-old mice were compared to 12-month-old animals. Absolute values of fat mass and lean mass are also included in the table, but given the difference in body weight between GHR−/− and WT mice, normalized values provide a better comparison between genotypes. Consistent with the literature (see Chapter 1), insulin levels were lower and adiponectin (total and HMW) levels were higher in the GHR−/− mice. However, there were no significant differences in the ratio of HMW to total adiponectin between genotypes. Regarding leptin levels, no differences were found between genotypes, probably due to the large variability detected. In addition, total and HMW adiponectin decreased significantly with age, especially in WT mice, as evidenced by a significant interaction of genotype × age. As observed for WT mice (Chapter 5), HMW adiponectin levels correlated closely with total adiponectin levels in GHR−/− mice ($r^2=0.74$, n=19, $P<0.001$, Figure 41).
Table 17

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Body weight (g)</th>
<th>Fat mass (g)</th>
<th>Lean mass (g)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Total Adip (µg/ml)</th>
<th>HMW Adip (µg/ml)</th>
<th>HMW Adip/total Adip (%)</th>
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<tr>
<td>GHR-/- mice</td>
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<tr>
<td>12 (n=13)</td>
<td>21.7 ± 2.0</td>
<td>9.8 ± 1.5</td>
<td>42.2 ± 2.9</td>
<td>9.8 ± 0.4</td>
<td>47.9 ± 2.5</td>
<td>0.87 ± 0.19</td>
<td>48.6 ± 12.7</td>
<td>38.5 ± 1.5</td>
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<tr>
<td>24 (n=6*)</td>
<td>16.5 ± 1.0</td>
<td>5.5 ± 0.7</td>
<td>32.7 ± 2.1</td>
<td>9.3 ± 0.3</td>
<td>56.5 ± 1.7</td>
<td>0.49 ± 0.05</td>
<td>19.6 ± 3.4</td>
<td>37.1 ± 2.5</td>
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<td>WT mice</td>
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<tr>
<td>12 (n=10)</td>
<td>38.8 ± 1.0</td>
<td>9.9 ± 0.8</td>
<td>25.3 ± 1.6</td>
<td>24.9 ± 0.5</td>
<td>64.5 ± 1.2</td>
<td>3.16 ± 0.58</td>
<td>31.4 ± 5.1</td>
<td>29.1 ± 2.0</td>
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<td>24 (n=6)</td>
<td>41.1 ± 3.2</td>
<td>8.1 ± 2.9</td>
<td>17.8 ± 5.3</td>
<td>28.3 ± 1.0</td>
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<td>22.9 ± 14.2</td>
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<td>ANOVA results†</td>
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All mice were male. Values are mean ± SE. (*) n=5 for insulin levels of the 24-month-old GHR-/- group. (†) Significant effects of genotype (G), age (A) or significant interaction of genotype × age (I) in a between-subjects two-way ANOVA (P<0.05). Abbreviations: GHR-/-, growth hormone receptor gene-disrupted; WT, wild-type; Adip, adiponectin, NS, not significant.
Correlations between hormone levels and fat mass

The relationships between fat mass and plasma levels of insulin, leptin, total and HMW adiponectin were evaluated in GHR-/- mice (Figures 42 and 43). These same correlations were analyzed for WT mice in Chapters 4 and 5. Insulin and leptin correlated significantly and positively to fat mass and percent fat mass in both GHR-/- and WT mice. In contrast, total and HMW adiponectin did not show correlations to fat mass or fat mass percentage in GHR-/- mice, whereas WT mice showed a significant correlation of fat mass and total adiponectin.

As observed for WT mice, GHR-/- mice showed varying relationships between variables with advancing age (Figure 43). Of special interest was the change in the correlation between adiponectin (total and HMW) and percent fat mass at 12 and 24 months of age in mice of both genotypes. It appears that adiponectin levels in mice only correlate with percent fat mass in old age. Moreover, in WT mice the correlation between
adiponectin (total and HMW) and fat mass was positive, whereas GHR-/- mice displayed negative correlations between those variables.
Figure 42. Correlations between plasma hormone levels and fat mass or fat mass percentage for combined age groups of each genotype. ○ GHR-/- mice; ■ wild-type mice. Solid lines fit GHR-/- mouse data points, dotted lines fit data for wild-type mice. Significant correlations are marked with (*).
Figure 43. Correlations between plasma hormone levels and fat mass percentage for each genotype and age group. ○ GHR-/- mice; ■ wild-type mice. Solid lines fit GHR-/- mouse data points, dotted lines fit data for wild-type mice. Significant correlations are marked with (*). Abbreviations: mo, months.
**Depot-specific weight and protein content**

WAT from inguinal, retroperitoneal, mesenteric and epididymal depots was dissected and weighed (Figure 44A). Each weight was then normalized to body weight, to enable comparison between genotypes (Figure 44B). Significant effects of genotype, depot, and age were observed. There were also significant interactions of genotype × depot, and depot × age. When the effects of genotype and age were tested in each depot using a two-way ANOVA, inguinal and retroperitoneal WAT showed significantly higher weight in the GHR-/- mice than controls ($P<0.001$ and $P=0.004$ respectively). On the other hand, the inguinal depot was the only depot that did not decrease with age; retroperitoneal ($P=0.003$), mesenteric ($P<0.001$), and epididymal ($P=0.002$) depots were all smaller in 24-month-old compared to 12-month-old animals.

Protein content (milligram of protein per gram of tissue) was measured in each depot (Figure 44C), displaying significant main effects of genotype, depot, age, and a significant interaction of depot × age. When analyzed in each depot, protein content was lower in GHR-/- than WT mice in inguinal ($P<0.001$), retroperitoneal ($P=0.010$) and mesenteric WAT ($P=0.028$). In addition, the retroperitoneal depot showed higher protein content in 24-month-old mice compared to 12-month-old animals ($P=0.001$). Interestingly, no differences between genotypes or age groups were observed in the protein content of the epididymal depot.
Figure 44. Weight, percent weight and protein content of the WAT depots in the four mouse groups. All mice were male; data shown are mean ± SE. For depot weight and percent depot weight, n=13 and 10 for 12-month-old GHR-/− and wild-type mice respectively, and n=6 for both 24 month-old GHR-/− and wild-type mice. For protein content, n=6 for all groups. ○ GHR-/− mice; ■ wild-type mice. Factors and interactions that showed significant effects on a three-way ANOVA for each variable are listed on the right. (A) WAT depot weight; (B) WAT depot percent weight; (C) WAT depot protein content.

Correlations between depot-specific weights and hormone levels

Given that in GHR-/− mice circulating insulin and leptin levels correlated significantly with fat mass (see above), and that individual WAT depot weights correlated with fat mass as well (inguinal, $r^2=0.90$; retroperitoneal, $r^2=0.92$; mesenteric,
$r^2=0.93$; epididymal, $r^2=0.95$; all $n=19$, $P<0.001$), the evaluation of correlations between depot weights and plasma insulin and leptin levels was carried out while controlling for total fat mass. These same correlations were evaluated for WT mice in previous chapters (for results see Chapter 5). The results for GHR-/- mice show significant correlations between insulin levels and inguinal ($r^2=0.55$; $df=15$, $P=0.001$), retroperitoneal ($r^2=0.71$; $df=15$, $P<0.001$), and mesenteric ($r^2=0.38$; $df=15$, $P=0.008$) WAT depots, whereas leptin correlated significantly with inguinal fat mass ($r^2=0.24$; $df=16$, $P=0.040$). Neither total nor HMW adiponectin levels correlated significantly with the weight of any of the WAT depots. In addition, and as opposed to what was observed in WT mice (Chapter 5), levels of the four hormones measured did not correlate among themselves in GHR-/- mice after controlling for fat mass (except for total and HMW adiponectin).

When 12 and 24-month-old mice were evaluated separately, 12-month-old GHR-/- mice showed similar results to the combined age groups, with significant correlations between insulin levels and inguinal ($r^2=0.71$; $df=10$, $P=0.001$), retroperitoneal ($r^2=0.76$; $df=10$, $P<0.001$), and mesenteric ($r^2=0.42$; $df=10$, $P=0.022$) WAT depot weights. However, leptin levels did not correlate to inguinal depot weight, in contrast to what was observed for grouped 12 and 24-month-old mice. The results for aged GHR-/- mice showed no significant correlations among the levels of the four hormones measured or between these hormones and WAT depot weights, except for total and HMW adiponectin ($r^2=0.85$; $df=3$, $P=0.026$). These results are markedly different from those obtained for aged WT mice (Chapter 5), where insulin correlated with
epididymal depot weight, and total adiponectin showed a negative correlation with the weight of the mesenteric depot.

**WAT proteome differences between GHR-/- and WT mice**

Proteins from WAT depots were homogenized and resolved by 2DE. The intensities of 169 spots were analyzed using a three-way ANOVA with depot as a within-subjects factor and age and genotype as between-subjects factors. Overall, 70 spots showed significant main effects or interactions of the evaluated factors (Figure 45). Whereas 38 spots showed effects of depot and 35 spots were influenced by age, only five spots showed significant differences between genotypes. The same trends were observed regarding interactions, where 18 spots showed interaction of depot × age but only one spot showed interaction of genotype × age and two showed interaction of genotype × depot. Finally, five spots showed a significant interaction of genotype × depot × age. Because genotype differences were the main interest in this project, we focused on the 12 spots that showed a main effect of genotype or significant interactions of genotype and the other factors (Figure 46). Using MS and MSMS, the 12 spots were identified, representing eight protein identities (Table 18). Spots described in previous chapters were assigned the same label used previously (e.g. spots 10a and 10b (ApoA-I) from Chapters 4 and 5 were labeled 10a and 10b in this Chapter), and new isoforms of already described proteins were assigned the same number but different letters (e.g. a new isoform of actin was labeled 5m). Table 19 summarizes the proteins identified, how they were affected by
genotype, depot, age or interaction of these factors, and their functions (cytoskeleton related, lipid transport, etc.).
Figure 45. Representative 2D-gel of WAT. A. Raw image. B. Same gel image showing 70 spots that displayed significant ($P<0.01$) intensity differences. Main effects are labeled using (o) for genotype; (□) for depot; and (x) for age. Significant interactions are labeled with capital letters: genotype × depot (A); genotype × age (B); depot × age (C); and genotype × depot × age (D). Dashed circles mark spots that only show significant interaction effects. Numbered labels correspond to spots that were described in previous chapters.
Figure 46. Representative 2D-gel of WAT showing a subset of 12 spots that displayed significant main effects of genotype or significant interactions of genotype and other factors ($P<0.01$). Spots are labeled as in Figure 45: main effects of genotype (○), depot (□), age (x); significant interactions of genotype × depot (A), genotype × age (B), depot × age (C), and genotype × depot × age (D). Numbered labels correspond to protein identities shown in Table 18.
Figure 47. Log intensity of protein spots that were significantly different between genotypes ($P<0.01$ in a three-way ANOVA with depot as a within-subjects factor and age and genotype as between-subjects factors, $n=6$ mice per group). Spot labels correspond to gel locations shown in Figures 45 and 46. Protein identities were obtained by MS and MSMS (Table 18). Factors and interactions that showed significant effects on each spot are listed on the right, below the protein name. Abbreviations: mo, months; Ehd2, EH
Figure 48. Log intensity of protein spots that showed significant interactions of genotype \(\times\) age (A) or genotype \(\times\) depot (B) \((P<0.01\) in a three-way ANOVA with depot as a within-subjects factor and age and genotype as between-subjects factors, \(n=6\) mice per group). Spot 18 also showed a significant interaction of genotype \(\times\) depot \(\times\) age. Spot labels correspond to gel locations shown in Figures 45 and 46. Protein identities were obtained by MS and MSMS (Table 18). Factors and interactions that showed significant effects on each spot are listed on the right, below the protein name (italicized text describes significant differences in a two-way ANOVA for each individual depot). Abbreviations: mo, months; Hb-β1, hemoglobin β-1, WT, wild-type; Ing, inguinal; Ret, retroperitoneal; Epi, epididymal. (○ GHR-/- mice; ■ wild-type mice).
Figure 49. Log intensity of protein spots that showed significant interactions of genotype × depot × age ($P<0.01$ in a three-way ANOVA with depot as a within-subjects factor and age and genotype as between-subjects factors, $n=6$ mice per group). The intensity of spot 18 also showed a significant interaction of genotype × depot × age and is depicted in Figure 48. Spot labels correspond to gel locations shown in Figures 45 and 46. Protein identities were obtained by MS and MSMS (Table 18). Factors and interactions that showed significant effects on each spot are listed on the right, below the protein name (italicized text describes significant differences in a two-way ANOVA for each individual depot). Abbreviations: mo, months; ApoA-I, apolipoprotein A-I; WT, wild-type; Ing, inguinal; Ret, retroperitoneal; Epi, epididymal. (○ GHR-/- mice; ■ wild-type mice).
**Table 18**

*Protein Identities of Spots Showing Intensity Changes between GHR-/- and WT Mice, Determined by MS and MSMS*

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<thead>
<tr>
<th>Spot Accession</th>
<th>Protein name</th>
<th>Unique peptides</th>
<th>Sequence coverage (%)</th>
<th>Score*</th>
<th>MSMS peptides matched</th>
<th>Error (Da)</th>
<th>m/z</th>
<th>Score*</th>
<th>Peptides detected by MSMS (up to 3 top scoring peptides)</th>
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<td>45</td>
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<td>F</td>
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<td>5h**</td>
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<td>Protein S100-A10 (S100 calcium-binding protein A10)</td>
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<td>R.EFPGFLENQKDPLA VDK.I</td>
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Although the NCBInr database was searched, accession numbers listed are from the SWISS-Prot database, so as to unify in an individual entry all the possible matches representing sequence variants of the same protein. (*) Peptide mass fingerprint (MS) scores and peptide MSMS scores are shown as [score/significance cutoff], and significant scores are shown in bold. See materials and
methods for a detailed description of the methodology used. (**) For most of the spots labeled “5” only the best scoring isoforms of actin are reported; however, more isoforms were matched with slightly lower scores. (†) The molecular weight of spots 24a and 24b and the MSMS fragments matched indicated that the spots contained a C-terminal fragment of EH domain-containing protein 2, however, MS matches included hits to peptides elsewhere in the protein (non-specific hits). Unique MS peptides matching only the C-terminal region of the protein were five, with a total coverage of 13% for 24a and four, with a total coverage of 10% for 24b. No MS matches were obtained using only those peptides, probably due to low coverage of the protein. This is a common problem encountered when searching for small protein fragments. (††) The molecular weight of spot 29 and the MSMS fragments matched indicated that the spot contained the 35kDa subunit of α-2 macroglobulin, however, MS matches included hits to peptides elsewhere in the protein (non-specific hits). Unique MS peptides matching only the 35kDa subunit (at the C-terminal region of the protein) were eight, with a total coverage of 5% for the whole protein, and 29% for the cleaved subunit. No MS matches were obtained using only those peptides, probably due to low coverage of the protein. As stated above, this is a common problem encountered when searching for small protein fragments in the databases.
### Table 19

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<th>Spot</th>
<th>Protein identity Structure</th>
<th>Significant effects observed</th>
<th>Function</th>
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<tr>
<td>5f,h,m</td>
<td>Actin</td>
<td>Genotype × depot (m); genotype × depot × age (f,h); depot (f,h,m); age (f,m); depot × age (f)</td>
<td>Microfilaments; muscle contraction</td>
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<td>24a-b</td>
<td>EH domain-containing protein 2 (Ehd2)</td>
<td>Genotype (a,b); depot × age (b)</td>
<td>Regulation of endocytosis</td>
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<td>30</td>
<td>S100-A10</td>
<td>Genotype</td>
<td>Binds Annexin A2 and may be involved in exocytosis</td>
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<td>10a-b</td>
<td>Apolipoprotein A-I (ApoA-I)</td>
<td>Genotype × depot × age (a,b); depot (a); age (a,b)</td>
<td>Cholesterol transport in HDL, anti-inflammatory functions</td>
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Table 19: continued

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<td>Genotype × depot; genotype × depot × age; age; depot × age</td>
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<td>Transthyretin (prealbumin)</td>
<td>Genotype; age</td>
<td>Binds thyroid hormones and RBP4</td>
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<td>29</td>
<td>α-2 macroglobulin</td>
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<td>Proteinase inhibitor</td>
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<td>15e</td>
<td>Hemoglobin subunit β-1</td>
<td>Genotype × age; age</td>
<td>Oxygen transport</td>
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</table>

Protein identities were obtained by MS and MSMS (Table 18). Spot labels correspond to gel locations shown in Figures 45 and 46. The listed factors and interactions include the specific isoform/s affected between parentheses.
*Correlations between hormone levels and depot-specific spot intensities in GHR-/- mice*

The circulating levels of insulin, leptin, and total or HMW adiponectin were tested for correlations with the intensities of the 12 spots that showed significant effects of genotype or related interactions. The analysis was performed by genotypes (GHR-/- and WT groups separately, Table 20) and for genotype groups further split according to age (Table 21). Only correlations with $r^2 > 0.50$ are shown. Interestingly, there were no such correlations when spot intensities were tested against insulin levels in GHR-/- or WT mice. This is probably due to the large variability observed in insulin levels among age groups in each genotype. Thus, only when each of these two mouse groups were split according to age were significant correlations to insulin found. Apart from some overlaps in the correlations of spot intensities with total and HMW adiponectin, there were no clear patterns in the results obtained.
Table 20

Significant Correlations between Plasma Hormone Levels and Spot Intensities in GHR-/- and WT Mice

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot</th>
<th>Depot</th>
<th>GHR-/- mice</th>
<th>WT mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb-β1</td>
<td>15e</td>
<td>Ret</td>
<td>(-) $r^2=0.53$, n=11, $P=0.011$</td>
<td></td>
</tr>
<tr>
<td>Total adiponectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I</td>
<td>10a</td>
<td>Mes</td>
<td>(-) $r^2=0.59$, n=11, $P=0.006$</td>
<td>(+) $r^2=0.71$, n=12, $P=0.001$</td>
</tr>
<tr>
<td>Annexin A5</td>
<td>18</td>
<td>Ret</td>
<td>(-) $r^2=0.69$, n=12, $P=0.001$</td>
<td></td>
</tr>
<tr>
<td>A2M</td>
<td>29</td>
<td>Epi</td>
<td>(-) $r^2=0.59$, n=12, $P=0.004$</td>
<td></td>
</tr>
<tr>
<td>HMW adiponectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>5m</td>
<td>Ret</td>
<td>(-) $r^2=0.57$, n=9, $P=0.018$</td>
<td></td>
</tr>
<tr>
<td>S100-A10</td>
<td>30</td>
<td>Mes</td>
<td>(-) $r^2=0.55$, n=11, $P=0.009$</td>
<td>(+) $r^2=0.61$, n=12, $P=0.003$</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>10a</td>
<td>Mes</td>
<td>(-) $r^2=0.62$, n=12, $P=0.002$</td>
<td></td>
</tr>
</tbody>
</table>

For all the correlations listed $P<0.05$ and $r^2>0.50$. Abbreviations: Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal; ApoA-I, apolipoprotein A-I; A2M, α-2 macroglobulin; Hb-β1, hemoglobin β-1. A plasma sample was not available for one of the GHR-/-
mice, therefore hormone levels could not be measured in this mouse and $n=11$ instead of 12 for the GHR-/- group. Also, spot 29 (S100-A10), which had very low molecular weight, had run out of the gels of two mesenteric WAT samples of 24-month-old GHR-/- mice. Thus, spot intensity could not be measured in these two samples and $n=9$ for the listed correlation regarding this spot.

Table 21

*Summary of Significant Correlations Found in GHR-/- and WT Mice when Age Groups Were Combined (All) or Split (12mo and 24mo)*

<table>
<thead>
<tr>
<th></th>
<th>GHR-/- mice</th>
<th>WT mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>12mo</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>(+) Mes, 5m</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+) Epi, 5f</td>
</tr>
<tr>
<td>ApoA-I</td>
<td></td>
<td>(-) Ing, 10a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-) Epi, 10a</td>
</tr>
<tr>
<td>Annexin A5</td>
<td></td>
<td>(+) Epi, 18</td>
</tr>
<tr>
<td>TTR</td>
<td></td>
<td>(+) Epi, 27</td>
</tr>
<tr>
<td>A2M</td>
<td></td>
<td>(+) Epi, 29</td>
</tr>
</tbody>
</table>
Table 21: continued

<table>
<thead>
<tr>
<th></th>
<th>GHR-/- mice</th>
<th>WT mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>12mo</td>
</tr>
<tr>
<td>Hb-β1</td>
<td>(-) Mes, 15e</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>(+) Mes, 5m</td>
<td></td>
</tr>
<tr>
<td>Ehd2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100-A10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin A5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb-β1</td>
<td>(-) Ret, 15e</td>
<td>(-) Mes, 15e</td>
</tr>
<tr>
<td>Total adiponectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 21: continued

<table>
<thead>
<tr>
<th></th>
<th>GHR−/− mice</th>
<th></th>
<th>WT mice</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>12mo</td>
<td>24mo</td>
<td>All</td>
</tr>
<tr>
<td>Ehd2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-) Ing, 24b</td>
<td></td>
<td>(-) Ret 24a</td>
</tr>
<tr>
<td>S100-A10</td>
<td></td>
<td>(-) Mes, 30</td>
<td></td>
<td>(-) Ing, 30</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>(-) Mes, 10a</td>
<td>(+) Ret, 10a</td>
<td></td>
<td>(-) Epi, 10a</td>
</tr>
<tr>
<td></td>
<td>(-) Mes, 10a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-) Mes 10b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin A5</td>
<td></td>
<td>(+) Ret, 18</td>
<td></td>
<td>(+) Ret, 18</td>
</tr>
<tr>
<td>TTR</td>
<td></td>
<td></td>
<td>(+) Epi, 27</td>
<td></td>
</tr>
<tr>
<td>A2M</td>
<td>(-) Ret, 29</td>
<td>(-) Epi, 29</td>
<td>(-) Epi 29</td>
<td></td>
</tr>
<tr>
<td>Hb-β1</td>
<td>(-) Mes, 15e</td>
<td>(+) Epi, 15e</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(+) Epi, 15e</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMW adiponectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>(-) Ret, 5m</td>
<td>(-) Epi, 5h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 21: continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>GHR-/− mice</th>
<th>WT mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>12mo</td>
</tr>
<tr>
<td>Ehd2</td>
<td>(−) Ing, 24a</td>
<td>(−) Ret, 24a</td>
</tr>
<tr>
<td></td>
<td>(−) Ing, 24b</td>
<td></td>
</tr>
<tr>
<td>S100-A10</td>
<td>(−) Mes, 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(+) Mes, 30</td>
<td></td>
</tr>
<tr>
<td>ApoA-I</td>
<td>(−) Mes, 10a</td>
<td>(−) Mes, 10a</td>
</tr>
<tr>
<td></td>
<td>(−) Mes, 10b</td>
<td></td>
</tr>
<tr>
<td>Annexin A5</td>
<td></td>
<td>(+) Ret, 18</td>
</tr>
<tr>
<td>A2M</td>
<td>(−) Epi, 29</td>
<td>(−) Epi, 29</td>
</tr>
<tr>
<td>Hb-β1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For all the correlations listed $P<0.05$ and $r^2>0.50$. Signs in parentheses indicate positive (+) or negative (−) correlations, followed by the depot where the correlation was found and the label of the spot involved. For each genotype, the first column shows results for combined age groups (All), whereas the other two columns display results for each individual age group (12 and 24 months of age). Abbreviations: mo, months of age; Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal; Ehd2, EH domain-containing protein 2; ApoA-I, apolipoprotein A-I; TTR, transthyretin; A2M, α-2 macroglobulin; Hb-β1, hemoglobin β-1.
Discussion

The goal of this study was to compare WAT depots and their age-related changes between GHR-/- and WT mice. Differences among WAT depots and their response to age have been described for WT mice in Chapters 4 and 5. The phenotype of GHR-/- mice (described in Chapter 1) includes a unique combination of obesity (mainly subcutaneous), with high insulin sensitivity and extended longevity. These characteristics offer an interesting comparison to WT mice and represent a convenient tool for the study of depot-specific influences on the pathophysiology of obesity, insulin resistance and aging.

Body weight, body composition and hormone level differences observed between GHR-/- and control mice were consistent with previous reports (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Bonkowski et al., 2006a; Coschigano et al., 2003; Egecioglu et al., 2006). GHR-/- mice were smaller in size, but showed higher percent fat mass and lower percent lean mass than WT mice. Plasma insulin levels were lower, and adiponectin (total and HMW) levels were higher in GHR-/- mice. Interestingly, even though HMW adiponectin levels are thought to represent the active form of this adipokine (Kaser et al., 2008; Waki et al., 2003; Whitehead et al., 2006), the ratio of HMW to total adiponectin showed no differences between the insulin sensitive GHR-/- and control mice. This is different from the increased ratio observed in 6-month-old GHR-/- mice compared to WT (Lubbers et al, unpublished results) (see Chapter 1). In addition, despite fat mass percentage being significantly higher in GHR-/- mice, there were no differences between genotypes in the levels of circulating leptin. Previous
studies reported increased leptin in non-fasted ~5 and 21-month-old GHR-/- males (Al-Regaiey et al., 2005; Egecioglu et al., 2006). However, after a 4-hour fast, 5-month-old GHR-/- mice had leptin levels similar to WT (Berryman et al., 2004). In this study, an 8-hour fast also resulted in leptin levels being similar in GHR-/- and WT mice. Thus, fasting appears to eliminate differences in leptin levels between the genotypes. On the other hand, as observed for WT mice, insulin, leptin and total adiponectin levels correlated significantly with fat mass and fat mass percentage in GHR-/- mice (Figure 42). Moreover, marked differences were observed when correlations between fat mass percentage and adiponectin (total and HMW) levels were tested separately in the different age groups. Not only were the correlations stronger in both groups of 24-month-old mice compared to 12-month-old animals, but the nature of the relationships were opposite between genotypes (positive in WT vs. negative in GHR-/- mice). Adiponectin levels are generally correlated negatively with fat mass (Kaser et al., 2008), consistent with the results observed in 24-month-old GHR-/- mice. As stated in Chapters 4 and 5, the negative association between body fat and adiponectin levels might be triggered by drastic increases in fat mass, as seen in ob/ob and db/db mice (Hu et al., 1996; Yamauchi et al., 2001); and in WT mice, the presence of a positive correlation (rather than a negative one) between adiponectin and fat mass might be due to their relatively low body fat percentage.

Individual WAT depot weights were compared between GHR-/- and WT mice after normalization to body weight. As expected, inguinal percent weight was significantly higher in GHR-/- mice than controls. Marked accumulation of WAT in the
SC region is a regular finding in GHR-/- mice (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Flint et al., 2006; Y. Li et al., 2003; Liu et al., 2004). The retroperitoneal depot also showed a significant increase in percent weight in GHR-/- mice, consistent with previous reports (Berryman et al., 2004; Egecioglu et al., 2006). In addition, retroperitoneal, mesenteric and epididymal WAT depot weights decreased with age in both genotypes, consistent with the decreased percent body fat observed in 24-month-old mice. Interestingly, changes observed with age and genotype in WAT depot weights were reciprocal to the changes in protein content in each depot. For instance, the protein content of all WAT depots except epididymal was lower in GHR-/- than WT mice. These reciprocal changes might be related to the size of the adipocytes present in each depot, because larger depot weights probably reflect larger adipocyte sizes (Marin et al., 1992a). Large adipocytes, which contain more lipid, might display relatively less protein content than smaller adipocytes. In fact, adipocyte sizes in inguinal and retroperitoneal WAT depots were shown to be larger in GHR-/- mice compared to WT at six months of age, with no difference in the epididymal depot (Kelder et al., 2007). Unfortunately, mesenteric adipocyte sizes were not evaluated in that study. Regarding effects of age, protein content in the retroperitoneal depot showed significantly higher levels in 24-month-old mice compared to 12-month-old animals. This increase was also observed for WT mice in Chapter 5 and might be a consequence of decreasing adipocyte size in this depot with advanced age. Cell size measurements in WAT depots of GHR-/- and WT mice as a function of age will help elucidate this matter.
Significant correlations between WAT depot weights and the four measured hormone levels in GHR-/- mice included levels of insulin and weights of the inguinal, retroperitoneal and mesenteric depots. However, when age groups were analyzed separately, these correlations were present only in 12-month-old GHR-/- mice. In addition, combined age groups in GHR-/- mice showed a significant correlation between leptin and inguinal depot weight. None of these correlations were observed in WT mice (Chapters 4 and 5), except for a weak but significant correlation between insulin levels and mesenteric depot weight. These differences suggest distinctions in the physiology of WAT depots in GHR-/- and WT mice, consistent with the described disparity in fat mass, insulin sensitivity and lifespan between genotypes (reviewed by List et al., 2010).

To clarify the molecular mechanisms behind these physiological distinctions of GHR-/- and WT mice, we resolved the proteomes of four WAT depots in 12 and 24-month-old GHR-/- mice and compared them to those of WT mice. Surprisingly, we found more spot intensities to be different among WAT depots and age groups than between genotypes. Genotype differences (including main effects of genotype or interactions of genotype and the other factors – age and depot) involved 12 spots, which were identified by MS and MSMS, resulting in eight protein identities. Three of these proteins were involved in cytoskeletal structure and rearrangements during endocytosis and exocytosis. Among them, two isoforms of actin (5f and 5h) showed high levels in the mesenteric depot of GHR-/- mice, similarly to what was observed in WT mice (Chapters 4 and 5). These two isoforms showed a significant interaction of genotype × depot × age, with a marked decrease in intensity in the retroperitoneal depot of aged compared to adult
GHR-/- mice. In Chapters 4 and 5, we have attributed the intensity changes of actin isoforms in WAT of WT mice to variations in the density of blood vessels in each depot. However, when compared to the changes in hemoglobin-β (spot 15e), the intensity levels in this protein show reciprocal changes to those of actin isoforms 5f and 5h. The reason for this negative association is not clear. If the actin isoforms detected emanated from smooth muscle walls of blood vessels, the variation in blood vessel density would be expected to affect these isoforms in the same way as hemoglobin-β. Therefore, the origin of actin isoforms 5f and 5h appears to be other than blood vessel walls. Another probable origin of actin isoforms are the cytoplasmic β and γ-actins present in all cells. In adipocytes, β and γ-actin were found to decrease during maturation (Spiegelman & Farmer, 1982). Following this premise, decreased actin levels could reflect decreased preadipocyte/adipocyte ratios in WAT depots with age. However, this ratio was shown to increase with age in the epididymal WAT depot of rats, with almost no change in the retroperitoneal depot (Kirkland et al., 1994). Therefore, expected changes in preadipocyte/adipocyte ratio do not seem to match the decreased actin levels detected in aged mice. It should be noted, however, that a third isoform of actin (5m), which showed a significant interaction of genotype × depot, was increased with age. Changes in this isoform might partially counteract the decreased levels of the other two isoforms, resulting in net changes of actin that are not significant. This third isoform of actin displayed lower molecular weight and, contrary to isoforms 5f and 5h, was low in mesenteric WAT relative to other depots in GHR-/- mice. Dissimilar variations in the
intensity levels of actin isoforms might represent differential regulation of their activity by post-translational modifications.

As described in Chapter 5, Ehd2 is a protein involved in endocytic processes and has been shown to regulate GLUT4 internalization from the cell membrane to endosomes in cultured adipocytes (Guilherme et al., 2004). Two spots affected by genotype were identified as C-terminal fragments of this protein. One of these (24a) was previously found to be lower in epididymal than inguinal WAT in aged WT mice (Chapter 5). In this study, intensity levels of this isoform were increased in GHR-/- mice relative to WT, with the second isoform (24b) exhibiting a similar behavior. As explained in Chapter 5, the C-terminal fragment contained in these spots includes the EH domain and a short acidic sequence of Ehd2, which are proposed regulatory regions, mediating binding to F-actin for endocytosis (Guilherme et al., 2004). The increased intensity of these spots in GHR-/- mice compared to WT might indicate lower GLUT4 endocytosis in GHR-/- mice, resulting in the prolonged presence of this transporter on the cell membrane. This is consistent with higher insulin sensitivity in GHR-/- than WT mice (Bonkowski et al., 2009; Bonkowski et al., 2006b; Guo et al., 2005; Liu et al., 2004; Masternak et al., 2005a; Panici et al., 2009). However, the activity of Ehd2 seems not to be specific for GLUT4, and might also affect endocytosis of other membrane transporters or receptors (Guilherme et al., 2004). Altered endocytosis has been observed in several aging tissues, with a reduction in aging mouse thyroid (Gerber et al., 1987), aging B-lymphocytes in mouse Peyer’s patches (Haq & Szewczuk, 1992), and aging sinusoidal endothelial cells in rat liver (Simon-Santamaria et al., 2010), and an increase in aging chondrocytes and
fibroblasts (Edwards et al., 1984). These reports suggest that endocytosis might be altered in advanced age in a tissue/cell-specific manner. Interestingly, decreased liver endothelial endocytosis has been partly attributed to enhanced deposition of collagen in old age (Simon-Santamaria et al., 2010). Given that GH activates collagen gene expression (Sjogren et al., 2007), altered endocytosis in WAT of GHR-/- mice could be related to a lower collagen synthesis resulting from the lack of GH action.

The protein S100A10 belongs to a family of proteins (S100) that bind annexins and participate in membrane reorganization processes (Rintala-Dempsey et al., 2008). In particular, S100A10 has been shown to interact with annexin A2, which has been suggested to regulate actin reorganization in premature senescence (Chretien et al., 2008). On the other hand, GH was found to downregulate several S100 proteins in human white blood cells (Chung et al., 2009). In this study, S100A10 was higher in WAT depots from GHR-/- mice than controls, which is consistent with absent GH signaling. Given that senescence is associated with actin reorganization events and to increased annexin A2 levels (Chretien et al., 2008), it is tempting to speculate that increased S100A10 levels in GHR-/- mice might be related to the increased lifespan in these animals. However, how increased S100A10 would result in beneficial modifications of the actin cytoskeleton is not clear.

A different annexin, annexin A5, showed significant interactions of genotype × depot, and of genotype × depot × age. Annexin A5, as other annexins, binds membrane phospholipids to allow for membrane reorganization, exocytosis and vesicular trafficking (Rintala-Dempsey et al., 2008). The role of annexin A5, however, has been more closely
related to the inhibition of blood coagulation, by displacing coagulation factors from negatively charged phospholipid membranes (Andree et al., 1992). Decreased annexin A5 levels were observed in the retroperitoneal depot of aged WT mice compared to aged GHR-/- mice, with a similar trend in the epididymal depot. As stated in Chapter 5, lower annexin A5 in WAT depots of 24-month-old WT mice might be related to the increased risk of cardiovascular disease associated with normal aging (Jousilahti et al., 1999). This would suggest that retroperitoneal and maybe epididymal WAT exert an important depot-specific influence on the regulation of hemostasis in old age. Maintenance of anticoagulant functions in GHR-/- mice are consistent with their extended lifespan.

An isoform of TTR showed higher levels in GHR-/- mice compared to WT, and as described for WT in Chapter 5, its levels increased at old age in both genotypes. As mentioned in Chapter 5, this protein is the main carrier of thyroid hormones in rodent plasma, and it also binds RBP4 in the circulation, decreasing its clearance by the kidney (The UniProt Consortium, 2009). A positive modulation of TTR gene expression by GH has been described in the liver, through the activation of hepatocyte nuclear factor 6 (Samadani & Costa, 1996). Thus, TTR levels would be expected to decrease in GHR-/- mice rather than increase. However, the change in this specific TTR isoform might not reflect the variation in total circulating TTR levels; we have previously reported decreased levels of a TTR isoform in serum samples of healthy humans treated with a GH releasing hormone analog (Sackmann-Sala et al., 2009), and eight isoforms of TTR were lower in transgenic bGH than WT mice in plasma at two to 19 months of age (Ding, 2009). Moreover, regulation of TTR expression in WAT could be different from that
observed in the liver. On the other hand, thyroid hormone levels are higher in male GHR-/- than WT mice (Westbrook et al., 2009); therefore, increased TTR levels might serve to buffer the increased thyroid hormone concentration in the circulation. Finally, serum levels of both TTR and RBP4 are indicators of nutritional status, so increased levels of TTR in GHR-/- mice could be related to their increased food intake and enhanced fat mass (Berryman et al., 2006; Coschigano et al., 2003; Egecioglu et al., 2006; Longo et al., 2010). However, this scenario does not apply for the increased TTR levels observed in old age, given that a decrease in fat mass was detected in aged mice of both genotypes.

The levels of αK2 macroglobulin (A2M) were decreased in GHR-/- mice compared to WT. This protein is an important inhibitor of proteinases in plasma, and has been suggested to decrease the clearance or regulate the function of cytokines by binding to them in the bloodstream (Tayade et al., 2005). A2M is an acute phase protein in rats (Dajee et al., 1998), but not so in humans (Tayade et al., 2005) or mice (The UniProt Consortium, 2009). Interestingly, increased A2M in human cultured fibroblasts and rat plasma has been related to increased senescence (Kondo et al., 2001; Ma et al., 2004; Mayot et al., 2007). In addition, human circulating A2M levels were found to increase from ~40 to ~80 years of age in healthy subjects (Ritchie et al., 2004; Wagner et al., 1982). Thus, low A2M levels in WAT of GHR-/- mice are consistent with increased lifespan in these animals. In addition, rat A2M gene expression was induced by GH in cultured fibroblasts (Campbell et al., 1995); thus, absence of GH signaling in GHR-/- mice might be partly responsible for their decreased A2M levels.
Two isoforms of ApoA-I (10a and 10b) were affected by an interaction of genotype × depot × age and showed similar intensity trends to those observed for hemoglobin-β (15e). This suggests that ApoA-I levels reflect circulating HDL particles in the bloodstream. HDL-cholesterol was shown to be lower in young male GHR-/- than WT mice (Egecioglu et al., 2006) and consistent with this, ApoA-I levels were lower in the retroperitoneal WAT depot (with a similar trend in mesenteric WAT) of adult GHR-/- than WT mice. The marked increase in ApoA-I and hemoglobin-β levels observed in retroperitoneal WAT of 24 compared to 12-month-old GHR-/- mice suggests important changes in blood flow with age in this depot, which might not occur in WT mice.

Regarding differences observed among depots or age groups, most of the spots that displayed significant effects of these variables have been previously described in Chapters 4 and 5. This means that WT and GHR-/- mice display substantial similarities in their WAT depots. For example, isoforms of CKB, α-fetoprotein, actin, CA-III, TIM, ApoA-I, MYL9, transgelin and hemoglobin-β showed similar depot-specific patterns of intensity in WAT of WT and GHR-/- mice. Likewise, spots displaying significant effects of age, including ENO, PDHE1-B, Idh3α, ATP synthase subunit β, CA-III, ApoA-I, ERp29, Sod1 and PPIase A, TTR, and hemoglobin-β, showed similar changes in WAT depots of GHR-/- and WT mice. Given the substantial differences observed in fat mass, insulin sensitivity and lifespan between genotypes, it was surprising to find more similarities than differences between GHR-/- and WT mice in the studied WAT depots. However, the physiological differences displayed between genotypes might depend not only on distinctive protein profiles, but also on the sizes of each WAT depot.
In summary, although most intensity changes among WAT depots and age groups were similar between GHR-/- and WT mice, important variations between genotypes were found in eight proteins (Figure 50). Three of these proteins are involved in cytoskeletal reorganization, and might influence the presence of receptors and transporters (such as GLUT4) on cell membranes, affecting insulin sensitivity. Changes in cytoskeletal architecture and lower A2M levels might also result in delayed senescence, prolonging longevity in GHR-/- mice. The characteristics of WAT depots of GHR-/- mice are summarized in Figure 51. In addition, hemoglobin-β and ApoA-I levels suggested blood flow changes with age, especially in the retroperitoneal depot of GHR-/- mice. At old age, this depot also displayed high amounts of the anticoagulant protein annexin A5, which might protect GHR-/- mice from cardiovascular disease. Additionally, significant changes in protein content with age in both genotypes were found in retroperitoneal WAT, suggesting that age-related changes might be more drastic in this depot than in the others. This is consistent with the greater age-related changes in preadipocyte gene expression found in retroperitoneal than epididymal WAT (Cartwright et al., 2010).
Figure 50. Summary of similarities and differences found in WAT of adult and aged GHR-/- and WT mice. Arrows indicate the direction of change (increase ↑, decrease ↓, no change ↔) for protein levels. Similarities include protein levels that are similar in both genotypes: the left column shows levels of proteins in specific depots compared to the other depots; the right column shows levels of proteins in 24-month-old mice compared to 12-month-old mice. Differences include protein levels that change between genotypes: both columns show levels of proteins in GHR-/- compared to WT mice. Arrows for weight and protein also show levels in GHR-/- relative to WT mice. Abbreviations: Ing, inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.
Figure 51. Schematic summary of WAT depot characteristics in 12 and 24-month-old GHR-/- mice. Arrows indicate the levels (high ↑, low ↓, variable ↔) of metabolic rate and stress in each depot. Sizes of upward arrows (↑) also indicate varying degrees of increased metabolism and stress, where smaller sizes represent lower levels than larger sizes (e.g. at 24 months, levels are high in all depots, however metabolism in inguinal WAT < retroperitoneal and mesenteric WAT < epididymal WAT). These values are based on measured protein levels of metabolic enzymes and stress-resistant proteins. GLUT4 presence on the cell membrane is based on the measured levels of EH domain-containing protein 2. Estimated collagen deposition in each WAT depot is low, based on the absence of GH signaling in GHR-/- mice. However, a slight increase in collagen deposition is predicted in these mice in advanced aged. Collagen fibers are depicted as blue lines surrounding the cells. Stars represent reduced senescence, as indicated by altered levels of senescence-related proteins (S100-A10, EH domain-containing protein 2, and α-2 macroglobulin). Insulin sensitivity has been estimated in each WAT depot and is represented using a colored scale. Predicted degrees of insulin sensitivity are based on the estimated levels of metabolism and stress, presence of GLUT4 on the membrane, amount of collagen deposition, and indicators of senescence. Abbreviations: WT, wild-type; mo, months; GLUT4, glucose transporter 4.
Future studies of WAT depot architecture in advancing age, looking at cell sizes, blood vessel density, and also cytoskeletal structure will complement these findings. Despite the varied cell types present in WAT (adipocytes, preadipocytes, immune cells, fibroblasts, neural cells, endothelial cells, etc.), none of the proteins found were indicative of variations in specific cell populations in the WAT depots of GHR-/- and WT mice. It would be interesting to establish whether the changes detected, such as in endo/exocytosis, affect all cells in WAT or only certain cell types.

In conclusion, WAT depots of GHR-/- mice displayed changes in the expression of proteins that have been involved in membrane protein recycling and senescence-related phenotypes. Despite an otherwise “normal” expression profile in each WAT depot studied, these differences might hold the key to the increased insulin sensitivity and extended longevity of GHR-/- mice (Figure 52).
Figure 52. Schematic summary of WAT depot characteristics in 12 and 24-month-old WT and GHR/-/- mice (combined Figures 40 and 51). Arrows indicate the levels (high ↑, low ↓, variable ↔) of metabolic rate and stress in each depot. Sizes of upward arrows (↑) also indicate varying degrees of increased metabolism and stress, where smaller sizes represent lower levels than larger sizes (e.g. at 24 months, levels are high in all depots, however metabolism in inguinal WAT < retroperitoneal and mesenteric WAT < epididymal WAT). These values are based on measured protein levels of metabolic enzymes and stress-resistant proteins. GLUT4 presence on the cell membrane is based on the measured levels of EH domain-containing protein 2. Estimated collagen deposition in each WAT depot is based on levels of GH signaling in each genotype (normal in WT and absent in GHR/-/- mice). Collagen fibers are depicted as blue lines surrounding the cells. Stars represent reduced senescence, as indicated by altered levels of senescence-related proteins (S100-A10, EH domain-containing protein 2, and α-2 macroglobulin). Insulin sensitivity has been estimated in each WAT depot and is represented using a colored scale. Predicted degrees of insulin sensitivity are based on the estimated levels of metabolism and stress, presence of GLUT4 on the membrane, amount of collagen deposition, and indicators of senescence. Abbreviations: WT, wild-type; mo, months; GLUT4, glucose transporter 4.
CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Three hypotheses were stated at the beginning of this project (Chapter 2) and the analyses performed to test each of them have provided interesting data about the physiology of WAT in 12 and 24-month-old WT and GHR-/- mice (summarized in Figure 52). Based on the information obtained and discussions presented, three main conclusions can be drawn.

**Hypothesis 1. Distinct adipose depots display differences in their protein expression profiles.**

As reviewed in Chapter 1, before this study several reports showed differences among WAT depots in the response to hormonal control (Wajchenberg, 2000), proportions of different cell types in each depot (Kirkland et al., 1994; Montague et al., 1997), vascularization and innervation (Cinti, 2001, 2005; Crandall et al., 1984), adipocyte sizes (Kelder et al., 2007; Pond et al., 1986; Zhang et al., 2002), adipokine production (Kershaw & Flier, 2004; Wajchenberg, 2000; Wozniak et al., 2009), and general protein expression (Peinado et al., 2010; Perez-Perez et al., 2009; Salgado-Somoza et al., 2010). Our data expand this information, providing adipocyte sizes (Figure 23) and protein content (Table 7) of four WAT depots in mice and indicating that the metabolic activity and antioxidant capacity of the epididymal and mesenteric depots are higher than those of the inguinal depot (based on levels of CKB and ATP synthase subunit d (involved in ATP synthesis); TIM, ENO and CA-III (possibly reflecting...
glyceroneogenesis); HSPβ1 and PRDX2 (antioxidant proteins) as shown in Figure 21). In addition, actin, MYL9 and transgelin levels (Figure 22) suggest higher blood vessel density in the mesenteric depot.

**Conclusion 1**: Individual WAT depots in the body are markedly different, showing variable protein content, adipocyte sizes, and expression of several proteins that suggest varying levels of blood vessel density, metabolic rate and cell stress.

**Hypothesis 2**: Proteomes of different adipose depots change with age in a depot-specific manner.

When 12 and 24-month-old WT mice were compared, most of the age-related changes observed in WAT were similar in the four WAT depots. These changes included increased aerobic glucose oxidation (based on high levels of ENO, PDHE1-B, Idh3α, ATP synthase subunit β; and low levels of CA-III isoforms 7d-e), which probably results in increased ROS production; high stress-resistant proteins (Sod1, ERp29, and PPIase); and enhanced lipolysis (based on increased levels of E-FABP) in aged mice as compared to younger adults (Figures 34 and 35). Increased lipolysis might also result in increased release of FFA and glycerol to the bloodstream (Figure 39), exacerbating insulin resistance in other tissues. In addition, increased protein content and decreased WAT depot weights observed in 24-month-old mice (Figure 29) were consistent with an age-related decrease in adipocyte size in the WAT depots analyzed, particularly in the retroperitoneal depot. On the other hand, depot-specific patterns that were unchanged
with age involved higher blood flow/blood vessel density in mesenteric WAT (based on levels of actin, MYL9, and transgelin, Figures 36 and 37) and higher TG turnover in epididymal WAT, with opposite levels in the inguinal depot (based on levels of CA-III isoforms 7a and 7f, Figure 36).

An interesting age × depot interaction was found for a regulatory fragment of Ehd2, which in aged WAT was significantly higher in the inguinal than the epididymal depot (Figure 38). Given the reported role of this protein in GLUT4 endocytosis, these results suggest higher GLUT4 presence in the cell membrane, promoting higher glucose uptake, and therefore higher insulin sensitivity in the inguinal depot and lower in the epididymal depot at older age. Therefore, even though all depots undergo changes suggestive of decreased insulin sensitivity, the degree of insulin resistance might still be lower in inguinal than epididymal WAT at old age (Figure 40).

**Conclusion 2: With minor exceptions, advanced age affects all WAT depots in a similar manner, reflecting decreased insulin sensitivity and increased oxidative damage.**

**Hypothesis 3.** Compared to WT, GHR-/- mice display different WAT proteomes in individual adipose depots that depend on the age of the animal and the specific adipose depot under consideration.

As observed previously (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Flint et al., 2006; Y. Li et al., 2003; Liu et al., 2004), SC WAT (in this study
represented by the inguinal depot) was significantly enlarged in GHR-/- mice compared to WT at both time points studied (Figure 44). According to the data presented in Chapter 4, this WAT depot presents low metabolic rate, and low oxidative damage (Figures 22 and 27). Therefore, the possibility exists that the enlarged mass of this depot accounts for some of the beneficial effects observed in GHR-/- mice, leading to enhanced insulin sensitivity and prolonged longevity. In addition, the fact that the gonadal (in our case epididymal) depot is mostly unchanged (Berryman et al., 2004; Berryman et al., 2006; Berryman et al., 2010; Egecioglu et al., 2006; Y. Li et al., 2003) and sometimes decreased (Coschigano et al., 2003; Flint et al., 2006) in GHR-/- mice (Figure 44), suggests that the ratio of SC to gonadal depot weight might be a good indicator of overall health in mice. This is consistent with transplantation studies performed by Tran et al. (2008), where SC fat transplanted into the intra-abdominal area improved insulin sensitivity in mice.

The specific differences found between GHR-/- and WT mice in their WAT proteomes, albeit a few, probably contribute to the increased insulin sensitivity and prolonged longevity of GHR-/- mice. In fact, functions of the affected proteins (α-K2 macroglobulin, S100-A10, Eh2 (Figure 47)) have been related to senescence processes, including actin reorganization and altered endocytosis, and may result in the prolonged presence of GLUT4 on the cell membrane, reflecting enhanced insulin sensitivity (Chapter 6, Figures 51 and 52). Other differences between genotypes in protein levels were observed mainly in retroperitoneal WAT of aged animals (ApoA-I, annexin A5 and hemoglobin-β (Figures 48 and 49)). These changes indicate effects of age in this depot,
probably regarding blood flow and/or adipocyte size that appear more marked in GHR-/- mice.

**Conclusion 3: Differences between WAT depots in GHR-/- and WT mice are minor.** The increased lifespan and insulin sensitivity found in GHR-/- mice might be due to altered levels of senescence-related proteins in WAT depots and to the larger mass of the SC depot.

Several considerations have to be noted regarding the data obtained and the conclusions drawn. First, as stated previously (Chapter 1), there are different ways to normalize protein levels measured in WAT (e.g. relative to tissue weight or per individual cell). This matter should be pointed out, given that the normalization used in these studies has critical impact on the results obtained. The analysis performed here took into consideration the abundance of each protein spot relative to an equal amount of total protein (150 µg) isolated from each depot. The fact that the WAT depots displayed different weights, protein content per gram of tissue, and mean adipocyte sizes implies that the results would have been different if expressed relative to these variables. To illustrate the different possibilities, the following examples involve a virtual protein spot “A” displaying equal intensity levels among the WAT depots in our study.

1) Total amount of A in each WAT depot: The abundance of A relative to whole depot weight is affected by the depot’s weight and its protein content per gram of tissue. For depots that have similar protein content per gram of tissue (e.g. inguinal and epididymal
in 12-month-old WT mice), the amount of A will depend on how many total grams of
tissue are present in the body. Given that there are more total grams of epididymal than of
inguinal WAT in the body of a 12-month-old WT mouse, the total amount of A would be
higher in the epididymal than the inguinal depot. Thus, even though A displayed equal
levels between the two depots in our analysis, if its abundance had been expressed as
total amount of A per depot, this protein would have been higher in epididymal than
inguinal WAT. Similar results would be obtained for depots displaying same weights but
different protein content per gram of tissue (e.g. inguinal and mesenteric WAT in 12-
month-old WT mice). Given that there are more milligrams of total protein per gram of
tissue in the mesenteric than the inguinal depot in 12-month-old WT mice, total content
of A would be higher in mesenteric than inguinal WAT in these mice. This is another
example where the abundance of A would be different between two depots if expressed
as total amount of A per depot.

2) Abundance of A per cell: Considering that cells other than adipocytes display similar
sizes in all WAT depots, the size of adipocytes probably represents the main factor
affecting the number of cells present in each gram of tissue. For example, at 12 months of
age in WT mice, epididymal WAT (of large adipocytes) probably displays fewer cells per
gram of tissue than inguinal WAT (of small adipocytes). Given that both of these depots
display similar protein content per gram of tissue, cells in epididymal WAT might
contain relatively more proteins than the cells in inguinal WAT. Therefore, the
abundance of A per cell would be higher in the epididymal than the inguinal depot in
these mice.
The examples above highlight the impact of normalization on the results obtained in this study. Following common use in the literature for WAT and other tissues, we chose to analyze the spot protein levels in each depot relative to a fixed amount of protein. However, WAT presents unique characteristics when compared to other tissues in the body, with variable size of depots as well as adipocytes. Therefore, other ways of normalization might be important to consider when studying WAT.

Another matter that deserves attention is the resolution capabilities of the technique used, which we estimate to involve approximately half the pool of proteins expressed in the WAT depots. As stated in Chapter 1, hydrophobic proteins, especially membrane receptors and lipid-droplet associated proteins are difficult to isolate and therefore usually not detected by 2DE. Furthermore, proteins of MW higher than 70 kDa or lower than 6 kDa and those with pI lower than 5 or higher than 8 were not resolved in our gels. Therefore, additional differences might exist among the studied WAT depots, age groups and genotypes, regarding proteins not resolved in our analysis.

Finally, it is worth noting that GLUT4 levels have not been measured in this study. The estimation of GLUT4 levels on the membrane of WAT cells is based on the levels detected of the C-terminal fragment of Ehd2 (see discussions in Chapters 5 and 6 and figures 40, 51 and 52). Although the hypotheses put forward are interesting, there are no direct data suggesting GLUT4 levels are different in the WAT depots and genotypes analyzed. At least in humans, mRNA levels of this transporter have been reported to be higher in subcutaneous than visceral WAT (Table 2). Future studies will shed light on
GLUT4 expression and permanence on the cell membrane, and glucose uptake in WAT depots of GHR-/− vs. WT mice.

Future directions

The results summarized above have provided interesting data about the physiology of WAT depots in aging WT and GHR-/− mice. This information opens up the horizon to new possibilities, with a myriad of rising new hypotheses about depot-, age- and genotype-specific molecular mechanisms that regulate WAT physiology. To continue this research, future attempts will be aimed at validating and complementing the results obtained.

Regarding the differences found among 12-month-old WAT depots of WT mice, future experiments will include immunohistochemical detection of PEPCK and HSPβ1. PEPCK is the rate-limiting enzyme in glyceroneogenesis, so its levels in WAT depots will show if this pathway is activated in the epididymal depot compared to the others, as suggested by our data (Chapter 4). Also, detection of HSPβ1 by immunohistochemistry will serve to confirm the proteomic results obtained and establish antioxidant activity differences among WAT depots. Furthermore, the immunohistochemical detection will show if these two proteins are expressed in adipocytes and/or in any other cell population of WAT (preadipocytes, endothelial cells, macrophages, etc.).

In addition, microarray analyses performed in our laboratory using 6-month-old mice have compared gene expression in (a) SC vs. epididymal WAT depots of GHR-/− mice, (b) SC WAT of GHR-/− mice vs. SC WAT of WT mice, and (c) epididymal WAT
of GHR-/- mice vs. epididymal WAT of WT animals. The mRNA expression results obtained in these experiments will be compared to the protein levels found in the present study. The activation of specific pathways will be compared, especially relative to glucose metabolism and stress-resistance genes.

To confirm the findings in WAT depots of 12 and 24-month-old WT mice that show increased aerobic glucose oxidation, the activities of several enzymes will be measured. Proteins involved in glycolysis, (e.g. pyruvate kinase), glyceroneogenesis (e.g. PEPCK or pyruvate carboxylase), and enzymes in the Krebs cycle will be assayed using available commercial kits (e.g. pyruvate kinase assay kit from Abcam, Cambridge, MA (ab83432)), or enzymatic assays reported in the literature. The determination of PEPCK activity will also complement the immunohistochemical detection in 12-month-old WT mice proposed above. On the other hand, to confirm that antioxidant activity is increased in aged WAT depots, the activity of Sod1 will be determined as well in 12 and 24-month-old WT mice (e.g. using an enzymatic activity assay kit available from Cell Biolabs, Inc., San Diego, CA (STA-340)). These data, in turn, will complement the information about levels of HSPβ1 in WAT depots to be obtained by immunohistochemistry in 12-month-old WT mice.

The increased protein content and decreased WAT depot weights observed in 24-month-old mice suggested an age-related decrease in adipocyte size in the WAT depots analyzed, particularly in the retroperitoneal depot. To confirm this notion, adipocyte sizes will be measured in the four WAT depots of 24-month-old WT mice for comparison to those of 12-month-old WT animals reported in Chapter 4. During dissection, portions of
WAT depots from 24-month-old mice were treated for histology, and stained slides are already available. Also, to test if the retroperitoneal depot in GHR-/- mice displays changes with age in its architecture that are different from those of the other depots in GHR-/- and from WAT depots in WT animals, adipocyte sizes will be measured in the four depots of 12 and 24-month-old GHR-/- mice. As is the case for the WAT samples of 24-month-old WT mice, histological slides of WAT depots are available for the two age groups of GHR-/- mice. These measurements will be compared to the results obtained in WT mice, to establish genotype-, depot- and age-related differences. Furthermore, given the association between collagen deposition and senescence, and between collagen and GH, all samples treated for histology will be stained for collagen using Masson’s trichrome staining (Figure 53).

Figure 53. Deposition of interstitial matrix in response to feeding CD2F1/Cr mice with mixed isomers of conjugated linoleic acid. Accumulation of collagen in the mammary glands was visualized as greenish-blue staining, using Masson’s trichrome, and can be seen in the WAT (E, arrows). Reproduced with permission from Russell, J.S., McGee, S. O., Ip, M. M., Kuhlmann, D., & Masso-Welch, P. A. Conjugated linoleic acid induces mast cell recruitment during mouse mammary gland stromal remodeling, J Nutr, (2007;137:1200-1207), American Society for Nutrition.
The presence of GLUT4 will be detected by immunohistochemistry in each depot to verify whether our estimations of this transporter’s levels in different depots, age groups, and genotypes are correct. Functionality of GLUT4 will also be tested by measuring glucose uptake in vivo in the different depots. These experiments involve administration of 2-deoxy-[U\(^{14}\)C]glucose to live mice, sacrifice of the animals 30 min after injection and measurement of radioactivity in the tissues harvested (Blair et al., 1995).

In addition, to establish the function of Ehd2 in WAT, and whether it plays a role in aging and insulin resistance, it would be interesting to generate whole body or WAT-specific gene-disrupted or transgenic mice involving this protein or its C-terminal fragment.

Finally, we plan to study the physiological impact of removal or transplantation of WAT in WT and GHR-/- mice. There are different possibilities to explore regarding these techniques, but they should be limited to the manipulation of inguinal, epididymal or retroperitoneal depots, which are more easily accessible during surgery. As stated above, Tran et al. (2008) have reported beneficial effects of transplantation of SC WAT to infrabdominal locations in healthy mice. Given the lower insulin sensitivity estimated for the epididymal depot, one possibility involves the surgical removal of this depot from young and older WT mice, to determine whether this leads to an improvement in insulin sensitivity and healthier aging. Another experiment could involve the transplantation of WAT from GHR-/- mice into WT mice, given that WAT from GHR-/- mice appears to be more insulin sensitive and show delayed senescence in all depots. In fact, some of these
studies are being performed by our collaborators (Masternak & Bartke, personal communication). Also, the effects of adding WAT to animals at the end of life (which display a decrease in fat mass) could be explored, to determine whether increasing fat mass is beneficial at this late age.

Overall, the results obtained in the present study have yielded ample information about depot-, age- and genotype-related differences in WAT of C57Bl/6J WT and GHR-/- mice. As stated above, these data give rise to countless new hypotheses about the mechanisms regulating depot-, age- and genotype-specific differences in WAT physiology. For example, it would be interesting to perform a similar analysis in WAT depots of other mouse models of obesity (diet-induced, ob/ob, etc.) or impaired insulin signaling (e.g. FIRKO mice). Also, further research could be aimed at determining whether specific proteins found to vary in this study could serve as targets for treatment or diagnosis of insulin resistance, oxidative damage, and other WAT-related pathologies related to aging. The possibilities are exciting and endless.
REFERENCES


APPENDIX A: POWERPOINT SLIDES FROM PRESENTATION FOR PH.D.

DISSERTATION DEFENSE

Depot-specific differences in white adipose tissue of wild-type and GHR-/- mice of different ages

Ph.D. dissertation defense
Lucila Sackmann Sala
August 5th, 2010

2 All the slides in the presentation have been generated with information covered in the main text of the dissertation. For general references and permissions to reproduce figures and tables, please see the main document.
Outline

• Background
  – The adipose organ
  – Adipose tissue in aging
  – The GHR-/- mouse
  – Proteomics

• Objectives, hypotheses and study design

• Summary of results
  – Study 1 (12mo WT → depot effect)
  – Study 2 (12 and 24mo WT → age effect)
  – Study 3 (12 and 24mo WT and GHR-/- → genotype effect)

• Conclusions and future directions

Background
The adipose organ

White adipose tissue
- Main energy store
- Secretion of adipokines with various functions
- Regulated by hormones and CNS

Adipose tissue functions

Appetite & energy balance
Hemostasis
Lipid metabolism
Blood pressure regulation

Reproduction
Insulin sensitivity & glucose homeostasis
Angiogenesis

Inflammation, immunity & acute phase response


Secretion products of WAT

- **Endocrine function**
  - Adiponectin
  - Resistin
  - Insulin-like growth factor-1/IGF-binding protein 3
  - Angiotensinogen
  - Etc.

- **Immunologic/metabolic actions**
  - Retinol binding protein 4
  - Leptin
  - Visfatin
  - Interleukins
  - Plasminogen activator inhibitor 1
  - Prostaglandins
  - Apolipoprotein E
  - Etc.


---

Cell populations in adipose tissue

- **Adipocytes (30-60% of total cells)**

- **Stromovascular fraction:**
  - Preadipocytes
  - Fibroblasts
  - Immune cells (macrophages and mast cells)
  - Vascular and neural tissue
  - Connective matrix

Cell populations and adipokines

- Leptin and adiponectin are mainly secreted from adipocytes
- >90% of remaining adipokines are also produced in stromovascular cells

- Isolated adipocytes → poor representation of the physiology of adipose tissue

- Variation in the amount of SV cells in different samples may explain:
  - High inter-individual variability
  - Depot-specific differences

WAT depots

- Subcutaneous (SC)
- Intra-abdominal
  - Visceral (VISC, drain into the portal vein)
    - Omental (small size in mice)
    - Mesenteric
  - Non-visceral (do not drain into the portal vein)
    - Retroperitoneal
    - Perirenal
    - Gonadal (epididymal/parametrial)
Human adipose depots

CT scans of human abdominal adipose tissue.
- Green = SC
- Blue = intra-abdominal (VISC + non-VISC)

Most studies use the term “visceral” to refer to all intra-abdominal fat

Adipose depots in mice

Regional differences in gene expression

- Most studies have focused on differences in gene expression between SC and VISC/intra-abdominal fat depots

- VISC/intra-abdominal obesity correlates with
  - cardiovascular disease
  - cerebrovascular disease
  - hypertension
  - glucose intolerance
  - type II diabetes
  - hyperlipidemia
  - overall increase in morbidity and mortality

---

Expression in SC ≠ VISC

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Protein</th>
<th>Sample</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin *</td>
<td>SC &gt; VISC</td>
<td>Isolated adipocytes</td>
<td>Human</td>
</tr>
<tr>
<td>Adiponectin *</td>
<td>SC &gt; VISC</td>
<td>Whole tissue</td>
<td>Human</td>
</tr>
<tr>
<td>LPL *</td>
<td>SC = VISC</td>
<td>SC &lt; VISC (activity)</td>
<td>Isolated adipocytes</td>
</tr>
<tr>
<td>HSL *</td>
<td>SC = VISC</td>
<td>Isolated adipocytes</td>
<td>Human</td>
</tr>
<tr>
<td>TNF-α *</td>
<td>SC = VISC</td>
<td>Isolated adipocytes</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>SC = VISC</td>
<td>Whole tissue</td>
<td>Human</td>
</tr>
<tr>
<td>PGE2</td>
<td>SC &lt; VISC (secreted)</td>
<td>Whole tissue</td>
<td>Human</td>
</tr>
<tr>
<td>IL-6 *</td>
<td>SC &lt; VISC (secreted)</td>
<td>Whole tissue</td>
<td>Human</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>SC &lt; VISC</td>
<td>Whole tissue</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>SC &lt; epl (secreted)</td>
<td>Isolated adipocytes</td>
<td>Rat</td>
</tr>
</tbody>
</table>

(*) Levels also vary according to adipocyte sizes
Regulation by hormones and the CNS

- Catecholamines $\rightarrow$ lipolysis VISC $>$ SC
- Insulin $\rightarrow$ lipogenesis/antilipolysis SC $>$ VISC
- Growth hormone (GH) $\rightarrow$ Lipolytic (depot-specific differences) $\rightarrow$ production of IGF-1 (insulin-like action)
  2 opposing actions: the results may be determined by GH serum levels

- Other hormones:
  - Glucocorticoids
  - Sex hormones
  - Thyroid hormones

Adipose tissue in aging

Aging:
- $\uparrow$ fat in lean tissues
- centralization of adiposity
- modified energy balance
- $\downarrow$ insulin response
- $\downarrow$ adipocyte size and $\uparrow$ adipocyte number
- $\downarrow$ WAT expandability
WAT expandability and aging

↓ WAT expandability (↓ adipocyte size) ➔ ↑ circulating fatty acids ➔ ↑ lipid accumulation in nonadipose tissues

↑ insulin resistance

lipotoxicity (↑ ROS and apoptosis)

aging

The GHR-/- mouse

- Disrupted GHR ➔ impaired GH signaling
- Dwarf phenotype
- ↓ IGF1
- ↓ fasting insulin and glucose
- ↑ insulin sensitivity
- ↑ lifespan (~2 years and 7 months, 30%)
- ↑ percent fat mass (obese)

- ↑ food consumption/ body weight
- ↑ metabolic rate (VO2)/ body weight
- ↑ serum leptin and adiponectin
- ↓ serum cholesterol, triglycerides and Apolipoprotein B
- ↓ bone mineral density and bone mineral content
Adipose depots in GHR-/- mice

Relative Mass of Adipose Depots Normalized to Body Weight in GHR-/- Mice Compared to WT Mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Subcutaneous</th>
<th>Retroperitoneal</th>
<th>Gonadal</th>
<th>Mesenteric</th>
<th>Interscapular</th>
<th>BAT</th>
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<td>F</td>
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<td>F</td>
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<tr>
<td>2-4</td>
<td>↑</td>
<td>↑</td>
<td>↑/↔</td>
<td>↔</td>
<td>↔</td>
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<td>↔</td>
</tr>
</tbody>
</table>

Berryman et al. (2004, 2006, 2010); Coschigano et al. (2003); Egocioglu et al. (2006); Flint et al. (2006); Li et al. (2003); Liu et al. (2004).

Proteomics
Two-dimensional gel electrophoresis (2DE)

- Advantages
  - Best validated technique available to study whole proteins
  - Available stains allow for quantitative measurements
  - Splice variants and post-translational modifications can be identified

- Disadvantages
  - Inadequate resolution of membrane proteins and those very acidic, basic or hydrophobic.
  - Low abundance proteins may be below detection limits (nanogram range)

Scott et al. (2005) Diab Vasc Dis Res, 2,2:54-60
2DE protocol: continued

Objectives, hypotheses and study design
Objectives

- To establish the differences in protein profiles present in WAT of distinct adipose depots in wild-type (WT) and GHR-/− mice
  - To explain the molecular mechanisms behind the development of insulin resistance, type 2 diabetes and cardiovascular disease generally observed in obese individuals
    - Putative diagnostic and therapeutic targets
  - To reveal physiologically relevant differences among WAT depots not associated to obesity

Hypotheses and study design

- **Hypothesis 1:** Distinct adipose depots display differences in their protein expression profiles
- **Hypothesis 2:** Proteomes of different adipose depots change with age in a depot-specific manner
Hypotheses... continued

- **Hypothesis 3**: Compared to WT, GHR-/- mice display different WAT proteomes in individual adipose depots that depend on the age of the animal and the specific adipose depot under consideration.

---

**Detailed study design**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Inguinal</th>
<th>Retroperitoneal</th>
<th>Mesenteric</th>
<th>Epididymal</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>12 mo</td>
<td>Mouse 1</td>
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<td>Mouse 6</td>
<td>Mouse 6</td>
<td>Mouse 6</td>
<td>Mouse 6</td>
</tr>
<tr>
<td>WT</td>
<td>24 mo</td>
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<td>Mouse 12</td>
<td>Mouse 12</td>
<td>Mouse 12</td>
<td>Mouse 12</td>
</tr>
<tr>
<td>GHR-/-</td>
<td>12 mo</td>
<td>Mouse 13</td>
<td>Mouse 13</td>
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<td>Mouse 13</td>
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<td></td>
<td>Mouse 18</td>
<td>Mouse 18</td>
<td>Mouse 18</td>
<td>Mouse 18</td>
</tr>
<tr>
<td>GHR-/-</td>
<td>24 mo</td>
<td>Mouse 19</td>
<td>Mouse 19</td>
<td>Mouse 19</td>
<td>Mouse 19</td>
</tr>
<tr>
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<td></td>
<td>Mouse 24</td>
<td>Mouse 24</td>
<td>Mouse 24</td>
<td>Mouse 24</td>
</tr>
</tbody>
</table>

**All mice were male**
Other parameters measured

- Body weight
- Body composition
  - Plasma levels:
    - Insulin
    - Leptin
    - Adiponectin (total and HMW)
- Adipocyte sizes (only for 12mo WT mice)

WAT depots studied

- Mesenteric
  - Lining the intestines
    (Intra-abd VISC)
- Retroperitoneal
  - Behind the kidneys
    (Intra-abd, non-VISC)
- Inguinal
  - Groin region
    (SC)
- Epididymal
  - Next to the testes
    (Intra-abd, non-VISC)
Epididymal WAT depot

- Largest intra-abdominal depot in mice
- Not present in humans
- Analogous role to human omental WAT
  - Protection of internal organs
  - Immune function (high production of inflammatory cytokines)
- No drainage into portal vein ($\neq$ omental)
- Adipocyte size Epi $>\$ SC ($\neq$ omental $<\$ SC)

Summary of results
Study 1

Depot weight and protein content
Representative gels

166 spots analyzed
166 spots analyzed

Spots displaying depot-specific differences

38 spots showed differences among WAT depots in 12mo WT mice
**Protein spot identities**

38 spots showed differences among WAT depots in 12mo WT mice (P<0.01)

15 protein identities

---

**Creatine kinase B**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Relative intensity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>Creatine kinase B-type (CKB)</td>
<td>↓</td>
<td>Phosphate transfer to creatine</td>
</tr>
<tr>
<td>2b</td>
<td>Creatine kinase B-type (CKB)</td>
<td>↓</td>
<td>Phosphate transfer to creatine</td>
</tr>
<tr>
<td>2c</td>
<td>Creatine kinase B-type (CKB)</td>
<td>↓</td>
<td>Phosphate transfer to creatine</td>
</tr>
<tr>
<td>2a-c</td>
<td>Creatine kinase B-type (CKB)</td>
<td>↓</td>
<td>Phosphate transfer to creatine</td>
</tr>
</tbody>
</table>

---

![Creatine kinase B diagram](image-url)
### ATP generation

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Ing</th>
<th>Ret</th>
<th>Mes</th>
<th>Epi</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a-c</td>
<td>Creatine kinase B-type (CKB)</td>
<td>↓/↔</td>
<td>↓/↔</td>
<td>↑</td>
<td>↓/↔</td>
<td>Phosphate transfer to creatine</td>
</tr>
<tr>
<td>13a-b</td>
<td>ATP synthase subunit d, mitochondrial</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td>↓</td>
<td>ATP synthesis</td>
</tr>
</tbody>
</table>

↑ ATP generation in the mesenteric WAT depot

### Glucose/lipid metabolism

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Ing</th>
<th>Ret</th>
<th>Mes</th>
<th>Epi</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Triosephosphate isomerase</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td>Glycolysis/glyceroneogenesis</td>
</tr>
<tr>
<td>6</td>
<td>Enolase</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↑</td>
<td>Glycolysis/glyceroneogenesis</td>
</tr>
<tr>
<td>7a-d</td>
<td>Carbonic anhydrase 3 (CA-III)</td>
<td>↓/↔</td>
<td>↔/↑</td>
<td>↓/↔</td>
<td>↑/↑</td>
<td>pH regulation; provide HCO₃⁻ for carbon-fixing reactions</td>
</tr>
</tbody>
</table>
Metabolic pathways

Glycolysis

Glyceroneogenesis

Glyceroneogenesis and lipolysis

Suggested to decrease lipotoxicity and improve insulin sensitivity, and to be the main mechanism of action of the antidiabetic drugs thiazolidinediones

(Tordjman et al, 2003; Cadoudal et al, 2005)
### Glucose/lipid metabolism

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Ing</th>
<th>Ret</th>
<th>Mes</th>
<th>Epi</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Triosephosphate isomerase</td>
<td>![down]</td>
<td>![leftrightarrow]</td>
<td>![leftrightarrow]</td>
<td>![up]</td>
<td>Glycolysis/glyceroneogenesis</td>
</tr>
<tr>
<td>6</td>
<td>Enolase</td>
<td>![leftrightarrow]</td>
<td>![down]</td>
<td>![leftrightarrow]</td>
<td>![up]</td>
<td>Glycolysis/glyceroneogenesis</td>
</tr>
<tr>
<td>7a-d</td>
<td>Carbonic anhydrase 3 (CA-III)</td>
<td>![down]/[leftrightarrow]</td>
<td>![leftrightarrow]/[up]</td>
<td>![down]/[leftrightarrow]/[up]</td>
<td>![leftrightarrow]/[up]</td>
<td>pH regulation; provide HCO₃⁻ for carbon-fixing reactions</td>
</tr>
</tbody>
</table>

↑ glyceroneogenesis and TG turnover in epididymal WAT  
↓ glyceroneogenesis and TG turnover in inguinal WAT

### Antioxidant proteins

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Ing</th>
<th>Ret</th>
<th>Mes</th>
<th>Epi</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a-b</td>
<td>Heat shock protein β1 (HSPβ1, heat shock 27 kDa protein, HSP27)</td>
<td>![down]</td>
<td>![leftrightarrow]/[up]</td>
<td>![leftrightarrow]/[up]</td>
<td>![up]</td>
<td>Anti-oxidant protein, immune response</td>
</tr>
<tr>
<td>12a-b</td>
<td>Peroxiredoxin-2 (thioredoxin peroxidase 1)</td>
<td>![down]</td>
<td>![leftrightarrow]</td>
<td>![up]</td>
<td>![leftrightarrow]</td>
<td>Anti-oxidant protein</td>
</tr>
</tbody>
</table>

↑ oxidative stress in mesenteric and epididymal WAT  
↓ oxidative stress in inguinal WAT
Cytoskeleton

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>Relative intensity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a-k</td>
<td>Actin (various forms)</td>
<td>↓/↔</td>
<td>Microfilaments; muscle contraction</td>
</tr>
<tr>
<td>11</td>
<td>Myosin regulatory light polypeptide 9 (MYL9)</td>
<td>-</td>
<td>Smooth muscle contraction</td>
</tr>
<tr>
<td>14</td>
<td>Transgelin (smooth muscle protein 22-α)</td>
<td>-</td>
<td>Actin cross-linking protein</td>
</tr>
</tbody>
</table>

↑ blood vessel density in mesenteric WAT

Summary - Study 1

**Mesenteric**
- ↑ ATP generation
- ↑ antioxidant proteins
- ↑ blood vessel density
- ↑ protein content
- ↑ metabolism
- ↑ stress

**Inguinal**
- ↓ glycereoneogenesis and TG turnover
- ↓ antioxidant proteins
- ↓ protein content
- ↓ metabolism
- ↓ stress

**Retropertoneal**
- Variable levels of metabolic enzymes and antioxidant proteins
- ↓ protein content
- ↔ metabolism
- ↔ stress

**Epididymal**
- ↑ glycereoneogenesis and TG turnover
- ↑ antioxidant proteins
- ↓ protein content
- ↑ metabolism
- ↑ stress
Study 2

Body weight and body composition

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Body weight (g)</th>
<th>Fat mass (g)</th>
<th>Fat mass (%)</th>
<th>Lean mass (g)</th>
<th>Lean mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (n=10)</td>
<td>38.8 ± 1.0</td>
<td>9.9 ± 0.8</td>
<td>25.3 ± 1.6</td>
<td>24.9 ± 0.5</td>
<td>64.5 ± 1.2</td>
</tr>
<tr>
<td>24 (n=6)</td>
<td>41.1 ± 3.2</td>
<td>8.1 ± 2.9</td>
<td>17.8 ± 5.3</td>
<td>28.3 ± 1.0</td>
<td>70.4 ± 4.6</td>
</tr>
</tbody>
</table>
Depot weight and protein content

- **Depot weight (kg)**
  - Inguinal
  - Mesenteric
  - Retroperitoneal
  - Epidermal

- **Protein content (mg/g of tissue)**
  - Inguinal
  - Mesenteric
  - Retroperitoneal
  - Epidermal

**Statistical Significance**
- Depot: $P<0.001$
- Age: NS
- Age*depot: NS

---

Spots displaying significant differences

- **Isoelectric point**
- **Molecular weight**

**Statistical Significance**
- Depot: $P<0.001$
- Age: NS
- Age*depot: $P=0.011$
Spots displaying significant differences

39 spots showed significant differences (P<0.01):
- depot (17)
- age (20)
- age*depot (4)

Spot showing age and depot effects

Log intensity

12 months old 24 months old
Inguinal Retroperitoneal Mesentric Epididymal

12 mo 10d 24 mo
Protein spot identities

39 spots showed significant differences (P<0.01):
- depot (17)
- age (20)
+ age*depot (4)

17 protein identities + 3 unknown

Ehd2 and E-FABP

EH domain-containing protein 2 (C-term):
- Modulates endocytosis of GLUT4
- C-term contains regulatory domains:
  - C-term ➔ regulation ➔ endocytosis ➔ GLUT4 on membrane ➔ glucose uptake

At 24mo ↓ glucose uptake in Epi and ↑ in Ing

Epidermal fatty acid binding protein:
- Minor lipid chaperone after A-FABP (AP2)
- Both FABPs promote lipolysis (through interaction with HSL)
  - [A-FABP + E-FABP] ➔ control lipolysis
- At 24mo ↑ lipolysis
Metabolic pathways activated at 24 mo

Summary - Study 2

**Aged mesenteric**
- ↑ blood vessel density
- ↑ protein content
- ↑ glucose oxidation
- ↑ ATP generation
- ↑ antioxidant proteins

**Aged retroperitoneal**
- ↑ protein content
- ↑ glucose oxidation
- ↑ ATP generation
- ↑ antioxidant proteins

**Aged ileal**
- ↓ glyceroneogenesis and TG turnover
- ↑ glucose uptake
- ↓ protein content
- ↑ glucose oxidation
- ↑ ATP generation
- ↑ antioxidant proteins

**Aged epididymal**
- ↑ glyceroneogenesis and TG turnover
- ↓ glucose uptake
- ↓ protein content
- ↑ glucose oxidation
- ↑ ATP generation
- ↑ antioxidant proteins
Study 3

Body weight and body composition

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Body weight (g)</th>
<th>Fat mass (g)</th>
<th>Fat mass (%)</th>
<th>Lean mass (g)</th>
<th>Lean mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHR-/- mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (n=13)</td>
<td>21.7 ± 2.0</td>
<td>9.8 ± 1.5</td>
<td>42.2 ± 2.9</td>
<td>9.8 ± 0.4</td>
<td>47.9 ± 2.5</td>
</tr>
<tr>
<td>24 (n=6)</td>
<td>16.5 ± 1.0</td>
<td>5.5 ± 0.7</td>
<td>32.7 ± 2.1</td>
<td>9.3 ± 0.3</td>
<td>56.5 ± 1.7</td>
</tr>
<tr>
<td>WT mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (n=10)</td>
<td>38.8 ± 1.0</td>
<td>9.9 ± 0.8</td>
<td>25.3 ± 1.6</td>
<td>24.9 ± 0.5</td>
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<td>17.8 ± 5.3</td>
<td>28.3 ± 1.0</td>
<td>70.4 ± 4.6</td>
</tr>
<tr>
<td>ANOVA results*</td>
<td>G</td>
<td>NS</td>
<td>G, A</td>
<td>G, A, I</td>
<td>G, A</td>
</tr>
</tbody>
</table>

Mean ± SE

* G= genotype, A= age, I= genotype*age (P<0.05, 2-way-ANOVA)
Depot weight and protein content

Spots displaying significant differences
Spots displaying significant differences

70 spots showed significant differences (P<0.01):

- age (35)
- genotype (5)
- depot (38)
- gen*depot (2)
- gen*age (1)
- depot*age (28)
- gen*depot*age (5)

Spots displaying significant effects of genotype (main or interactions)

12 spots showed significant effect of genotype or interactions of genotype with other factors (age and/or depot) (P<0.01):

- age (8)
- genotype (5)
- depot (4)
- gen*depot (2)
- gen*age (1)
- depot*age (1)
- gen*depot*age (5)
Protein spot identities

12 spots showed significant effect of genotype or interactions of genotype with other factors (age and/or depot) (P<0.01):
- age (8)
- genotype (5)
- depot (4)
- A: gen*depot (2)
- B: gen*age (1)
- C: depot*age (1)
- D: gen*depot*age (5)

α-2 macroglobulin (A2M)

- Inhibitor of proteinases in plasma - binds cytokines, may regulate their action
- Senescence in humans and rats => A2M (A2M increases from age 30-80 years in humans)
- GH induces A2M in rat fibroblasts

>> ↓ A2M may be related to lack of GH and prolonged longevity in GHR-/- mice
**S100-A10**

- S100 proteins bind annexins and participate in membrane reorganization
- S100-A10 interacts with annexin A2 (annexin A2 \(\uparrow\) in senescence, may regulate actin reorganization in premature senescence)
- GH downregulates several S100 proteins in human white blood cells

>> \(\uparrow\) S100-A10 may be related to lack of GH and prolonged longevity in GHR/- mice

**Edh2 and endocytosis**

- Altered rates of endocytosis in aging tissues (thyroid, B-lymphocytes, liver endothelial cells, chondrocytes and fibroblasts)
- Possible role of collagen deposition – GH induces collagen expression

>> GHR/-: \(\uparrow\) Edh2 (and \(\downarrow\) collagen) \(\rightarrow\) \(\uparrow\) GLUT4 on the membrane (\(\uparrow\) glucose uptake)
Summary of Study 3

**Depot-specific proteomes**
- Mesenteric VAT:
  - ↑ CKB
  - ↑ Actin
  - ↑ MYL9
  - ↑ Transgelin
- Epididymal VAT:
  - ↑ CA-III
  - ↑ TrioseP isom
- Inguinal VAT:
  - ↓ CA-III
  - ↓ TrioseP isom

**Effects of age**
- ↑ Enolase
- ↑ Pyruvate dehydrogenase
- E1 β
- ↑ Isocitrate [NAD] dehydrogenase α
- ↑ ATP synthase subunit β
- ↓ Carbonic anhydrase 3
- ↑ ER resident protein 29
- ↑ Cu/Zn superoxide dismutase
- ↑ Peptidyl-prolyl isomerase

**Endo/exocytosis and senescence**
- ↑ EH domain-containing protein 2
- ↑ S100-A10
- ↓ α2-macroglobulin
- (and differential regulation of actin isoforms)
- ↑ Transthyretin
- ↑ Apolipoprotein A-I
- ↑ Annexin A5
- ↑ Hemoglobin-β
- (*) more marked in retro at 24mo

<table>
<thead>
<tr>
<th>Ing</th>
<th>Ret</th>
<th>Mes</th>
<th>Epi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>↑↑</td>
<td>↑</td>
<td>← ↔</td>
</tr>
<tr>
<td>Protein</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Depot and age differences in WT mice

- **Inguinal**
  - 12mo: ↓ metab, ↓ stress
  - 24mo: ↑ metab, ↑ stress

- **Retroperitoneal**
  - 12mo: ↔ metab, ↔ stress
  - 24mo: ↑ metab, ↑ stress

- **Mesenteric**
  - 12mo: ↑ metab, ↑ stress
  - 24mo: ↑ metab, ↑ stress

- **Epididymal**
  - 12mo: ↑ metab, ↑ stress
  - 24mo: ↑ metab, ↑ stress

Insulin sensitivity: Higher (Green) to Lower (Red) with Collagen fibers.
Depot and age differences in GHR-/- mice

Inguinal: GLUT4 ↓ metab ↓ stress
Retroperitoneal: ↔ metab ↔ stress
Mesenteric: ↑ metab ↑ stress
Epididymal: ↑ metab ↑ stress

12mo

GHR-/- mice

Inguinal: ↑ metab ↑ stress
Retroperitoneal: ↑ metab ↑ stress
Mesenteric: ↑ metab ↑ stress
Epididymal: ↑ metab ↑ stress

24mo

Depot and age differences in GHR-/- mice

Inguinal: GLUT4 ↓ metab ↓ stress
Retroperitoneal: ↔ metab ↔ stress
Mesenteric: ↑ metab ↑ stress
Epididymal: ↑ metab ↑ stress

12mo

GHR-/- mice

Inguinal: ↑ metab ↑ stress
Retroperitoneal: ↑ metab ↑ stress
Mesenteric: ↑ metab ↑ stress
Epididymal: ↑ metab ↑ stress

24mo

Reduction in senescence
Insulin sensitivity: Higher → Lower
Collagen fibers

72
73
Depot, age, and genotype differences in WAT

Conclusions and future directions
Hypotheses tested and conclusions drawn

- **Hypothesis 1:** Distinct adipose depots display differences in their protein expression profiles
  - **Conclusion 1:** Individual WAT depots in the body are markedly different.

- **Hypothesis 2:** Proteomes of different adipose depots change with age in a depot-specific manner
  - **Conclusion 2:** With minor exceptions, advanced age affects all WAT depots in a similar manner, reflecting decreased insulin sensitivity and increased oxidative damage.

- **Hypothesis 3:** Compared to WT, GHR/- mice display different WAT proteomes in individual adipose depots that depend on the age of the animal and the specific adipose depot under consideration
  - **Conclusion 3:** Differences between WAT depots in GHR/- and WT mice are minor. The increased lifespan and insulin sensitivity found in GHR/- mice might be due to altered levels of senescence-related proteins in WAT depots and to the larger mass of the SC depot.

Future directions

- **Confirm main differences among WAT depots**
  - Immunohistochemical staining for PEPCK (glyceroneogenesis) and HSPB1 (antioxidant activity)
  - Compare results with microarray data (SC and epi GHR/- and WT)

- **Confirm main differences between age groups**
  - Enzymatic activity assays: Pyr kinase (glycolysis); PEPCK or Pyr carboxylase (glyceroneogenesis); Krebs cycle enzymes; SOD1 (antioxidant activity)
  - Histological analysis: adipocyte sizes (24 mo) and collagen stain
Future directions

- Confirm differences between GHR-/- and WT mice
  - Immunohistochemical staining for GLUT4
  - Measure glucose uptake in WAT depots of WT and GHR-/- mice
  - Histological analysis: adipocyte sizes (12 and 24 mo) and collagen stain

- Look into the mechanisms of aging and insulin resistance
  - Transgenic mouse: WAT specific overexpression of Ehd2 or its C-term fragment

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