Organ Specific Lipid Imaging and Quantification with WETSEM™ Technology: A New Tool for Early Evaluation of Drugs for Metabolic Diseases

▶ The Need for Detection of Lipid Accumulation in Tissues

Type 2 diabetes (T2DM) is a disease in which the body develops a resistance to the hormone insulin, which is secreted by the beta-cells in the pancreatic islets of Langerhans. Initially, the resistance to insulin, primarily at the level of the muscle, liver and adipose tissues, results in higher circulating insulin levels. Chronic hyperinsulinemia then eventually leads to beta-cell exhaustion, a drop in circulating insulin levels and the subsequent elevation in blood glucose levels (1). The causes of T2DM are not fully understood, but it is generally accepted that it is both polygenic and heterogeneous in nature with both genetic and environmental factors such as obesity, diet and inadequate exercise playing important roles in the pathogenesis of the disease.

While T2DM has been traditionally associated with high blood glucose levels, the role of lipids in the pathogenesis of obesity and diabetes continues to gain import. The prevalent use of the term "diabesity" serves to underscore the gaining recognition of the close link between obesity and diabetes. Insulin resistance serves as the common underlying pathology and is believed, in part, to be the result of dysfunctional lipid metabolism, resulting in the accumulation of lipids in the various insulin-sensitive tissues to the point of being toxic to the cell (2).

Longitudinal studies in the Zucker Diabetic Fatty (ZDF) rat show that plasma free fatty acid levels rise before blood glucose levels and the animals become diabetic (2). The elevations in circulating levels of free fatty acids (FFAs) and triglycerides (TG), and a buildup of fat in several tissues including the adipose tissue, muscle, liver and beta-cells, are attributed to a dysregulation of lipid metabolism. Whether this dysregulation in lipid metabolism is a cause or result of insulin resistance is yet to be fully understood.

While the quantity of lipid accumulated into the cellular space is an important factor, equally important is the nature of its intracellular distribution and association with other cellular organelles, such as the mitochondria. The ability to study intracellular lipid accumulation and distribution patterns has been technologically hampered, especially where it relates to the skeletal muscle (3). Based on WETSEM™ technology, QuantomiX Ltd. recently introduced a microscopy tool that provides a practical method to accurately assess intracellular lipid morphology and quantity in skeletal muscle and in other tissues, such as liver and adipose tissue. With this new tool, cells or tissues undergo minimal sample preparation, insuring lipid droplets are preserved intact and avoiding artifacts caused by standard preparation procedures. Thus, high resolution lipid imaging and quantification is achieved in a rapid, accurate and convenient manner (4, 5).

▶ Intracellular Lipid Accumulation in Skeletal Muscle

The accumulation of lipids in the muscle plays an integral role in the pathogenesis of insulin resistance, in particular, the levels of intramyocellular lipids (IMCL) versus the metabolically inert lipids in the extracellular space (EMCL). Recent studies using magnetic resonance spectroscopy (MRS) to measure IMCL levels have contributed greatly to the understanding of the interplay between IMCL levels, insulin resistance and diabetes (6, 7). IMCL levels are known to be positively correlated with obesity and negatively correlated with insulin sensitivity (8). The correlation between IMCL and insulin resistance is independent of body mass index (BMI) and fasting glucose levels, which therefore

cannot be used as estimates of IMCL. This indicates that IMCL is a stronger and conceivably an earlier predictor of insulin resistance. Moreover, IMCL levels are increased among first-degree relatives of type 2 diabetic individuals and related to the expression of insulin resistance in this high-risk group, even prior to clinical manifestation of the disease (9).

Intriguingly, exercise training increases insulin sensitivity and IMCL levels (10-12). This data, while seemingly paradoxical, is physiologically consistent with the importance of fatty acid as an energy source during exercise. Fatty acid metabolism is an important aspect of triglyceride accumulation and a major portion of the lipid metabolism pathway resides in the mitochondria. In recent studies, Kelly and colleagues have shown that following weight loss and exercise intervention in obese non-diabetic individuals, the IMCL droplet size decreased together with increases in the number of mitochondria, and improved insulin sensitivity. Therefore, the hypothesis has been proposed that smaller droplets are more accessible to oxidation by mitochondria and therefore represent a better metabolic condition (13). Parallel studies by Shulman and colleagues using ^{31}Phosphorus spectroscopy demonstrate that the rates of cellular ATP production are 30% lower in the muscle from insulin resistant offspring of patients with diabetes, relative to normal subjects (9).

Hence, changes in IMCL content and mitochondria together appear to contribute to the development of insulin resistance, while their changes occur early in the pathogenesis of the diseases. Whether these changes are a cause or consequence of insulin resistance is not yet fully known, due in part to the paucity of reliable technology and tools to study changes in these two cellular parameters. WETSEM™, for the first time, affords investigators the ability to address and answer this long outstanding question.

Traditionally, biochemical methods have been used to measure muscle levels but these techniques only provide data on total muscle lipid content which includes IMCL and EMCL. MRS allows for a separation of IMCL from EMCL and has the advantage of being non–invasive in nature. However, its cost and limited access diminish its use. Moreover, it does not allow for the assessment of the relative cellular topography and lipid droplet size assessment in the muscle fibers.

As illustrated in Figure 1, data from WETSEM™ demonstrate that the technology is highly sensitive in allowing for high-resolution imaging of IMCL. It shows IMCL accumulation in the muscle of diet-induced diabetes sand rats (Psammomys Obesus) after three weeks on a high-energy diet (B), vs. basal conditions when fed low energy diet (A).
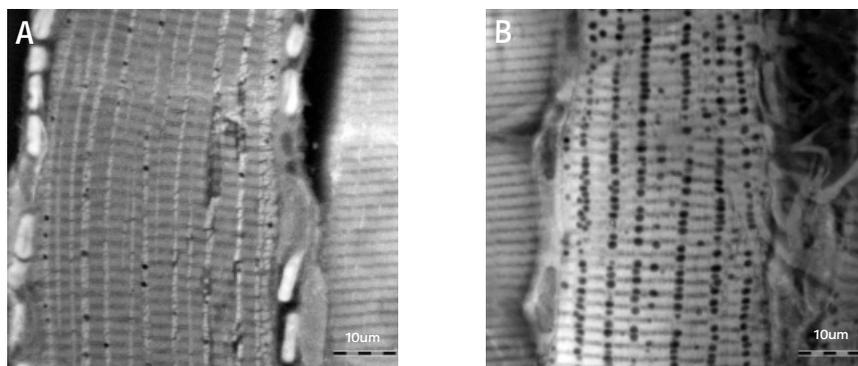

Figure 1: Longitudinal view of skeletal muscle from the P. Obesus rodent model of diet-induced diabetes, showing increased lipid droplet quantity and size (shown in black) in the hyperglycemic animal (B), compared with the control (A). Bar 10 micrometer. In collaboration with Prof. N. Kaiser, Hadassah Medical Center, Israel.

Proprietary QuantomiX analysis software was used to quantify the IMCL, as lipid density across muscle fibers (figure 2, left panel) and as droplet size distribution (figure 2, right panel). The experimental groups included four animals per time point (basal, one week, and three weeks on a high-energy diet, as well as two days on a low energy diet following the three weeks on the high-energy diet).
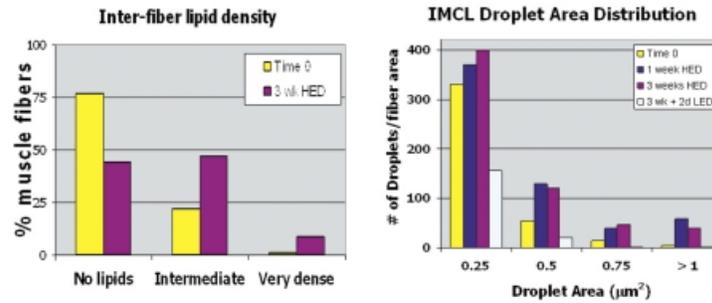

Figure 2: IMCL data analysis. Left: IMCL lipid density across muscle fibers at basal and hyperglycemic conditions. Right: IMCL droplet area distribution. Data was collected for at least 50 muscle fibers per animal.

Lipid density across muscle fibers demonstrates that the transition to hyperglycemia came in conjunction with increases in the percentage of muscle fibers containing lipids. A closer look at the size distribution of the IMCL droplets (right panel) indicates that the development of hyperglycemia (blue and purple bars) is characterized by the appearance of larger lipid droplets. The differentiation between control and hyperglycemic animals is clearly apparent as the IMCL droplet size increases (the X axis). Diet restriction for two days after the animals developed hyperglycemia (white bar) reversed their hyperglycemia back to normal, and accordingly, their IMCL state. These results emphasize the tight coupling of lipid accumulation with the appearance of systemic hyperglycemia, specifically in lipid droplets of larger size within myocytes.

Figure 3 illustrates how WETSEM™ also provides simultaneous visualization of cellular mitochondria and the spatial relationship with the lipid droplets, exemplifying the importance of the mitochondria in cellular lipid metabolism and insulin resistance.
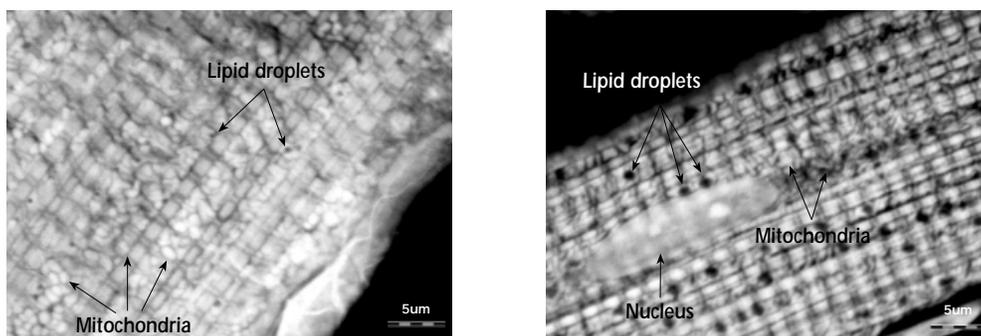

Figure 3: Lipid and mitochondria in skeletal muscle

Given the role of lipid accumulation in the pathogenesis of diabetes, the ability to accurately measure IMCL levels could provide for an early biomarker and thereby enhance the ability to detect and treat the disease at an earlier stage.

Insulin resistance at the level of the liver leads to higher glucose output and hence fasting hyperglycemia (2). Changes in the liver fat content play an important role in hepatic steatosis and the pathogenesis of T2DM (14). Nonalcoholic fatty liver disease (NAFLD) consists of two stages: 1. fat accumulation within hepatocytes (steatosis), and 2. A local inflammatory response, (NASH or nonalcoholic steatohepatitis). At present, little is known about the natural history or the underlying vulnerability of individual patients in terms of developing progressive liver disease after initial steatosis. While total liver lipid content can be measured biochemically as well as by MRS, the distribution of this lipid cannot be adequately assessed. Furthermore, undetectable changes, while small, may play an early role in the hepatic toxicity induced via the administration of development compounds. Demonstration of the ability to accurately visualize and quantify liver lipid content is illustrated in Figure 4.
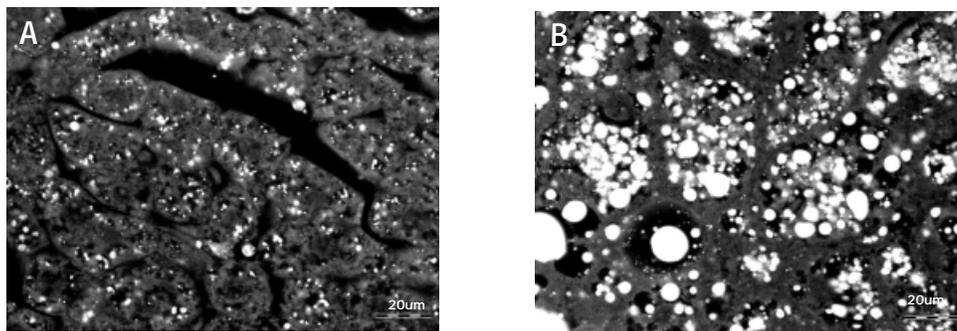

Figure 4: Lipid accumulation in liver tissue from a control wild type (A) and an obese ob/ob mouse (B). Samples were stained with Osmium. Lipid bodies are seen in white. Bar: 20 micrometer.

Image analysis software was used to quantify lipid accumulation in liver tissue. As shown in Figure 5, lipid content in ob/ob mice was seven fold compared to age matched control mice.
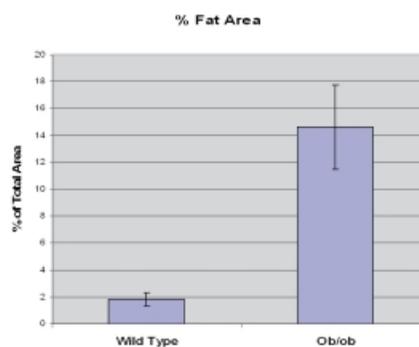

Figure 5: Average lipid accumulation in liver of three control and three ob/ob mice. Analysis performed on images taken from two different lobes, three sections per lobe.

QUANTOMIX™

While tissue mass can be assessed non-invasively using dexa and MRI, these techniques cannot determine the qualitative nature of the adipose tissue changes. This is especially important given that small adipocytes are more insulin sensitive and therefore more beneficial than large adipocytes in terms of their positive effects on insulin sensitivity.

At the preclinical level, where adequate amounts of adipose tissue can be harvested from mice and rodents, the Coulter counter serves as effective and fairly easy method to assess adipocyte size distribution (15). However, in clinical studies where fat biopsies limit the amount of adipose tissue available for cell sizing studies, the Coulter counter is not the method of choice. Here, WETSEM™ provides an easy and reliable method to determine adipose tissue morphology and adipocyte size distribution.

Figure 6 illustrates the use of WETSEM™ to assess adipose tissue morphology at high resolution.
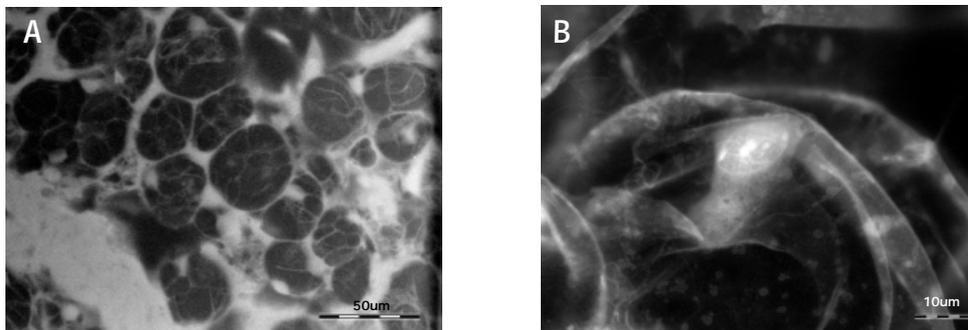

Figure 6: White adipose tissue (unstained) from normal mice, showing low (A, bar 50 micrometer) and high (B, bar 10 micrometer) magnifications, useful for cell size determination and precise tissue morphology, respectively. In collaboration with Prof. S. Cinti, Ancona University, Italy

Figure 7 demonstrates how cell sizing can be assessed in tissue biopsies of human adipose tissue using this technique.
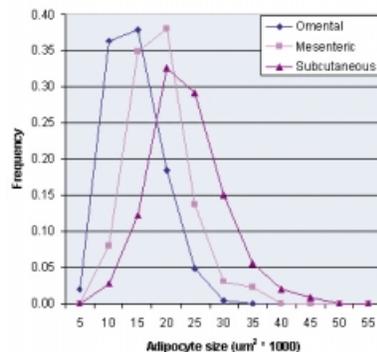

Figure 7: Adipocyte size distribution from three different fat depots (mesenteric, omental, and subcutaneous) from an obese patient, showing that different fat depots are characterized by different cell size. Data was collected from at least 200 cells per sample. In collaboration with Prof. B. Corkey and Dr. C. Apovian, Boston University Obesity Research Center.

QUANTOMIX™

▶ Lipid droplet Accumulation in Adipocytes

The differentiation of pre-adipocytes into mature fat cells is a complex process controlled by the interplay of intracellular factors and signals from the environment. Mature adipocytes are characterized by a high content of lipogenic enzymes which facilitate the synthesis and cytoplasmic storage of massive amounts of triglycerides. When given the proper hormonal conditions, 3T3-L1 pre-adipocytes undergo differentiation in culture, acquiring morphological and biochemical characteristics of adipocytes. The monitoring of triglyceride accumulation throughout their differentiation pathway can serve as a phenotypic marker for their maturation state and therefore be useful in cell-based assays to evaluate intervention effects in vitro.
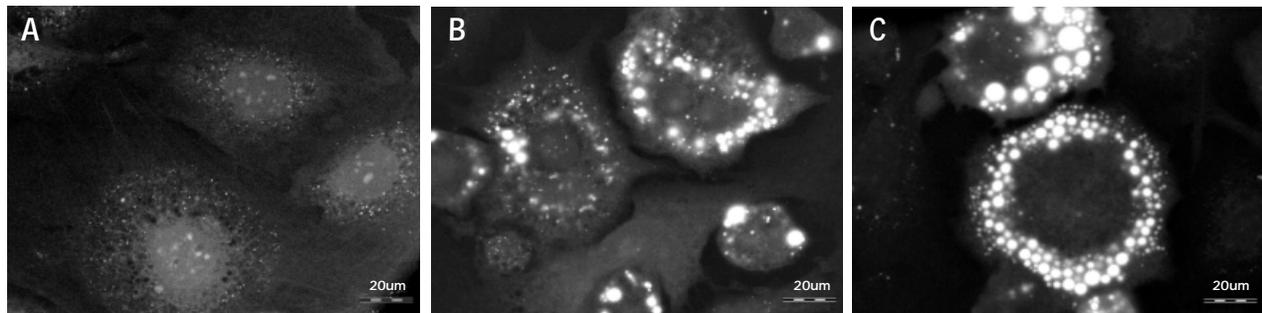

Figure 8: 3T3-L1 cell differentiation (stained with Osmium), showing increased lipid content as cells mature in culture. (A) Basal conditions. (B) 3 days differentiation in culture. (C) 6 days. Bars, 20 micrometer.

▶ New Opportunities for Drug Development and Beyond

These and other studies have shown the wide-range utility of WETSEM™ technology in lipid research. WETSEM™ is the only comprehensive solution for lipid imaging and quantification for the evaluation of metabolic diseases. Along with its ability to accurately analyze tissue morphology, this capacity opens new opportunities for researchers and drug developers alike.

References

1. De Fronzo RA: The triumvirate: ß-cells, muscle, liver: a collusion responsible for NIDDM. Diabetes 37:667–687, 1988
2. McGarry JD. Banting Lecture 2001 - Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 51:7-18, 2002
3. Kelly, DE, Goodpaster, BH. Skeletal Muscle Triglyceride - An aspect of regional adiposity and insulin resistance. Diabetes Care 24:933–941, 2001
4. Thiberge S, Nechushtan A, Sprinzak D, Gileadi O, Behar V, Zik O, Chowers Y, Michaeli S, Schlessinger J, Moses E. Scanning electron microscopy of cells and tissues under fully hydrated conditions. Proc Natl Acad Sci USA 101:3346-3351, 2004
5. Thiberge S, Zik O, Moses E. An apparatus for imaging liquids, cells, and other wet samples in the scanning electron microscope. Rev Scientific Instruments, 75:2280-2289, 2004
6. Shen W, Mao XL, Wang ZM, Punyanitya M, Heymsfield SB, Shungu DC. Measurement of intramyocellular lipid levels with 2-D magnetic resonance spectroscopic imaging at 1.5 T. Acta Diabetologia. 40:S51-S54, 2003
7. Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, and Stein DT. Measurement of intracellular triglyceride stores by 1H spectroscopy: validation in vivo. Am J Physiol Endocrinol Metab 276: E977–E989, 1999
8. Machann J, Haring H, Schick F, Stumvoll M. Intramyocellular lipids and insulin resistance. Diab. Ob. Metab. 6:239-248, 2004
9. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with Type 2 Diabetes. N Engl J Med 350:664-671, 2004
10. de Combaz J, Schimitt B, Ith M, Decarli B, Diem, P, Kreis R, Hoppeler H, Boesch C. Postexercise fat intake repletes intramyocellular lipids but no faster in trained than in sedentary subjects. Am J Physiol. 281:R760–R769, 2001
11. Hoppeler H, Fluck M. Plasticity of skeletal muscle mitochondria: structure and function. Med Sci Sports Exerc. 35(1):95-104, 2003
12. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. J Clin Endocrinol Metab 2001; 86: 5755–5761
13. He J, Goodpaster BH, Kelley DE. Effects of weight loss and physical activity on muscle lipid content and droplet size. Ob Res 12:761-769, 2004
14. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic Fatty Liver Disease - A Feature of the Metabolic Syndrome. Diabetes 50:1844–1850, 2001
15. Tchoukalova YD, Harteneck DA, Karwoski RA, Tarara J, Jensen MD. A quick, reliable, and automated method for fat cell sizing. J. Lipid Res. 44:1795–1801, 2003

QUANTOMiX™