Adipose Organ Development and Remodeling

COMPREHENSIVE PHYSIOLOGY

Adipose Organ Development and Remodeling

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ABSTRACT

During the last decades, research on adipose tissues has spread in parallel with the extension of obesity. Several observations converged on the idea that adipose tissues are organized in a large organ with endocrine and plastic properties. Two parenchymal components: white (WATs) and brown adipose tissues (BATs) are contained in subcutaneous and visceral compartments. Although both have endocrine properties, their function differs: WAT store lipids to allow intervals between meals, BAT burns lipids for thermogenesis. In spite of these opposite functions, they share the ability for reciprocal reversible transdifferentiation to tackle special physiologic needs. Thus, chronic need for thermogenesis induces browning and chronic positive energy balance induce whitening. Lineage tracing and data from explant studies strongly suggest other remodeling properties of this organ. During pregnancy and lactation breast WAT transdifferentiates into milk-secreting glands, composed by cells with abundant cytoplasmic lipids (pink adipocytes) and in the postlactation period pink adipocytes transdifferentiate back into WAT and BAT. The plastic properties of mature adipocytes are supported also by a liposecretion process *in vitro* where adult cell in culture transdifferentiate to differentiated fibroblast-like elements able to give rise to different phenotypes (rainbow adipocytes). In addition, the inflammasome system is activated in stressed adipocytes from obese adipose tissue. These adipocytes die and debris are reabsorbed by macrophages inducing a chronic low-grade inflammation, potentially contributing to insulin resistance and T2 diabetes. Thus, the plastic properties of this organ could open new therapeutic perspectives in the obesity-related metabolic disease and in breast pathologies. © 2018 American Physiological Society. *Compr Physiol* vol_number: page_range, year.

Didactic Synopsis

Major teaching points

- Adipocytes are lipid rich cells.
- White adipocytes, organized in white adipose tissue (WAT), store energy allowing intervals between meals.
- Brown adipocytes, organized in brown adipose tissue (BAT), burn lipids for thermogenesis.
- Both WAT and BAT are contained in a dissectible organ.
- The adipose organ shows a similar composition in all mammals, including humans.
- The prevalent tissue is WAT, but BAT is present in several depots.
- The adipose organ is provided with dense vascular and nerve supply especially in BAT.
- WAT and BAT are interconvertible tissues to satisfy specific physiologic requirements: whitening to allow energy storing when the energy balance is chronically positive and browning when thermogenesis is chronically required.
- Remodeling is mainly due to plasticity of parenchymal noradrenergic nerve fibers and hormonal factors.
- During pregnancy-lactation white adipocytes convert reversibly to alveolar cells (pink adipocytes).
- The plastic properties of adipose organ allow energy repartition among three vital needs: metabolism, thermogenesis, and lactation.

Introduction

Obesity and T2 diabetes are linked diseases and the term diabesity has been coined (129). Diabesity has a series of cardiovascular and neoplastic consequences representing major health problems and one of the primary cause of death for humans not only in industrialized countries but also in developing areas of the world (531,971). The inevitable continuous civilization process of humans has enhanced exponentially the two main causes underlining this pathology: low-cost food availability and reduction of physical activity with inevitable positive energy balance outcome.

Therapeutic approaches attempted in the last decades was oriented mainly to drugs with anorectic effects with the goal of calorie intake reduction. This approach has failed mainly because of important secondary effects such as depression and

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suicide. This was quite expected considering the importance of food intake rewording effect especially in the main range of age affected by diabesity (i.e., 50-70 years old) (256, 333, 1018). One therapeutic procedure able to obtain long-term effects is bariatric surgery (844), but this is not possible for most people.

Basic science studies are of paramount importance to avoid surgery by discovering the cause and prospecting innovative therapeutic approaches (187).

Adipose tissues anatomy, physiology, and pathology are central for understanding diabesity but have been neglected by scientists for many years. In the last 50 years, many scientific achievements were performed in this field including the discovery that adipose tissues are organized to form a large organ with impressive remodeling capacities. These remodeling properties could represent a valid objective for future diabesity therapeutic approach. This comprehensive review outlines some of the basic aspect of remodeling properties of adipose organ.

Adipose Organ Anatomy

Histology

White adipocyte

For historical reasons, the definition of a cell as an adipocyte is simply related to the conspicuous amount of lipids in the cytoplasm of the cell, without any reference to its function (153, 622, 848).

White adipocytes are capable of storing and releasing highly energetic molecules known as fatty acids. Their morphology is ideal to obtain the highest concentration of molecules used to perform biological energy in a minimum space. As a matter of fact, the best geometrical shape for this achievement is the spherical shape and adipocytes are spherical cells that assume this shape at very early stages of development. Their anatomy is quite simple because about 90% of their volume is occupied by a single droplet of triglycerides (lipid droplet) (Fig. 1). The lipid droplet is contained into the cytoplasm but it is not bounded by a plasma membrane, as are other organelles. At the interface with cytoplasm, the lipid droplet presents a dense line, visible by electron microscopy (Fig. 1), due to the presence of proteins playing important roles in the physiology of fatty acids traffic and metabolism (38, 74, 356, 821). The most studied of these protein is perilipin 1, a lipid droplet coat protein that protect the lipid droplet from lipolysis. It is quite specific for adipocytes and lipolytic enzymes require its phosphorylation to act on triglycerides (821). Fsp27 is another of these lipid droplet proteins and has recently been shown to be essential for unilocular arrangement of cytoplasmic lipids in white adipocytes (352, 1016).

Mitochondria of adipocytes are thin elongated and form a branched network well visible by confocal microscopy techniques (240). Electron microscopy shows their short and randomly oriented cristae. The endoplasmic reticulum is composed of elongated linear cisternae in the rough part (RER) and small cisternae in the smooth part (SER). This last is often in contact with the lipid surface, suggesting functional relationships (Fig. 1).

The Golgi complex is usually small and located in the perinuclear area. A series of microvesicles of various sizes are often diffuse in the thin rim of cytoplasm.

Several pinocytotic vesicles are present at the cell membrane. On the outer side of the cell membrane, a distinct external lamina is always well visible by electron microscopy (153, 622, 848). Its molecular composition is similar to that of other mesenchymal derived cells, such as muscle cells. Immunohistochemistry data showed the presence of collagen IV, laminin, and heparan sulfate in correspondence of the external lamina in mature human subcutaneous adipocytes (691). In this work, we did not detected immunostaining for fibronectin in line with *in vitro* data showing a strong decrease of fibronectin synthesis during adipocyte development, but other authors have found fibronectin in adult adipocytes contained in bovine intermuscular areas (619).

On the outer side of external lamina fibrillary collagen is well visible by both transmission and high-resolution scanning microscopy (336, 550, 618, 674, 792).

The nucleus is crescent shaped squeezed by the lipid droplet at periphery of the cell both in mature and in developing adipocytes even from very early stages of their development.

The size of mature adipocytes is variable and depends on the technique used for the study. Data from fresh fixed fat are about 20% to 30% larger (169, 282) than those usually reported from paraffin embedded tissue. In adult lean small mammals, the largest adipocytes are usually located in the abdominal subcutaneous and epididymal visceral fat with sizes ranging from 50 to 70 μm in diameter.

The smallest white adipocytes are usually found in other visceral depots or in intraorgan locations (bone marrow, parathyroid gland, parotid gland, heart, thymus, gastrointestinal tract, skeletal muscles, and lymph nodes). Their size is variable but roughly it can be considered around 2/3 of that of subcutaneous white adipocytes (165).

White adipose tissue

White adipocytes are organized to form a tissue called white adipose tissue (WAT) (Fig. 2) because its color is white in small mammals but in humans, it appears yellow (169). The tissue is composed of several cell types, including vascular cells (endothelium, pericytes, muscle cells, and adventitial cells) and nerve cells (Schwann cells, perineural cells, and neurons) (166). Fibroblasts and immune cells such as macrophages, eosinophils, lymphocytes, and mast cells are the most common interstitial cells found in WAT. Furthermore, a variable amount of adipocyte precursors is usually present in the pericapillary space (552, 683, 878).

All these elements have peculiar ultrastructural features and can be easily distinguished by electron microscope

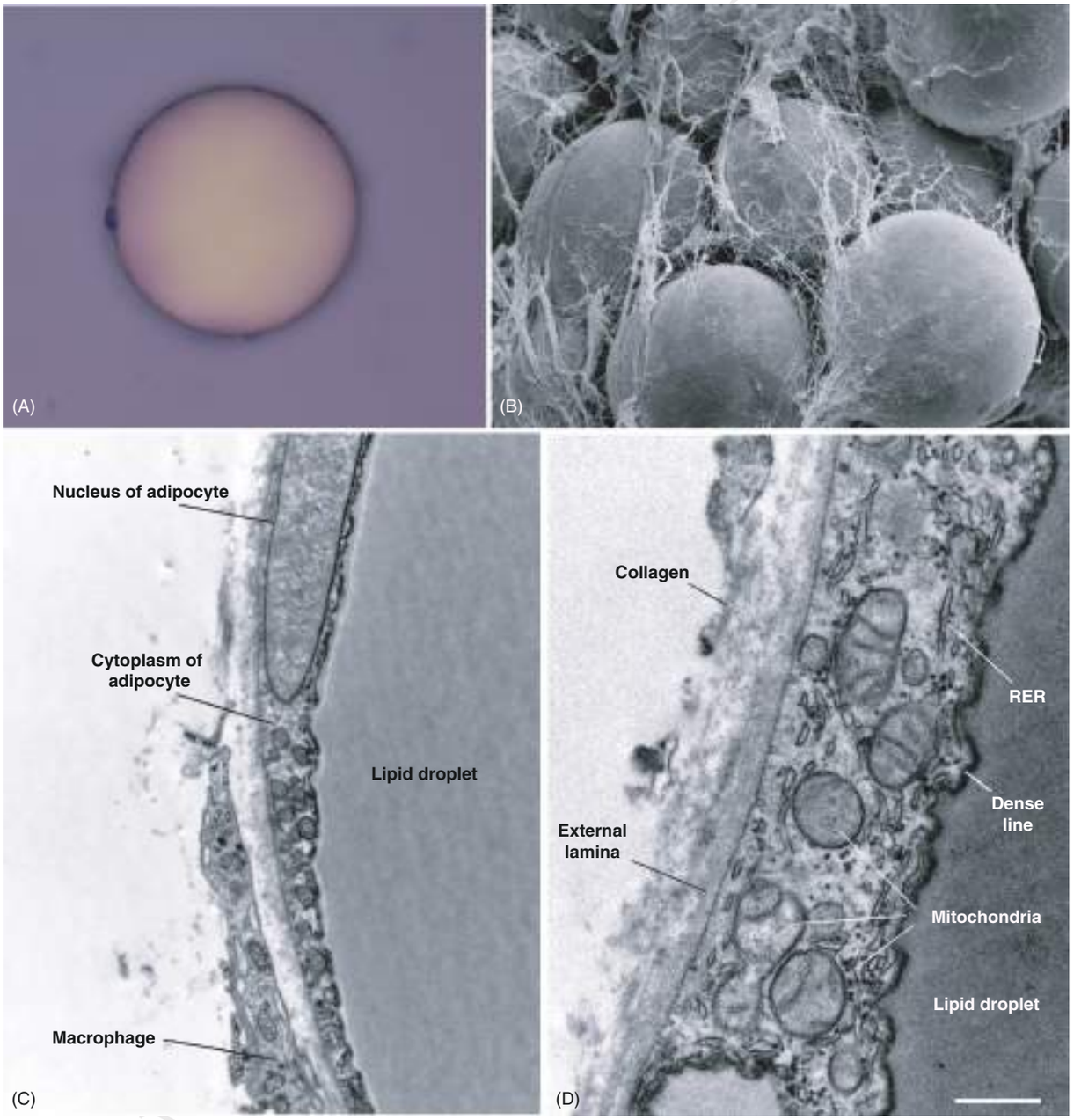

Figure 1 Light microscopy (A) and electron microscopy morphology of isolated human adipocyte (A), murine white adipose tissue (B, scanning electron microscopy), human subcutaneous adipocytes (C and D, transmission electron microscopy). Bar: in A, 15 μm; in B, 20 μm; in C, 1.2 μm; and in D, 0.3 μm. B adapted, with permission, from (167).

analyses (171, 180, 424, 622). Fibroblasts are spindle shaped and characterized by abundant dilated RER. They are responsible for extracellular matrix formation including collagen fibrils. Collagen VI seems to be highly represented in human fat (676, 797) Macrophages are irregularly shaped with elongated sinuous, thin cytoplasmic projections, abundant primary and secondary lysosomes, and well-developed Golgi complex. Eosinophils show classic granules with a discoid crystal in an equatorial position. Lymphocytes have a very high nucleus/cytoplasmic ratio with characteristic nuclear heterochromatin. Mast cells are roundish, enriched with large cytoplasmic dense and structured granules. All five of these cell types lack an external membrane, which is instead a distinctive feature of adipocytes and adipocyte precursors (281, 622). This last cell is also characterized by high nucleus/cytoplasmic ratio and poorly differentiated

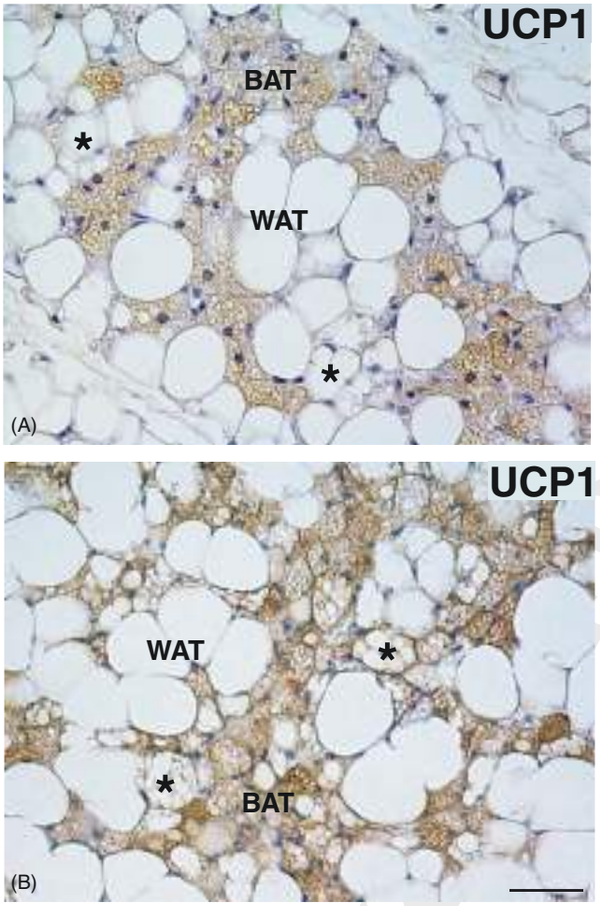

Figure 2 UCP1 immunohistochemistry of mixed (white and brown adipose tissues) areas of murine (A) and human (B) adipose organ. Bar: in A, 35 μm; and in B, 50 μm.

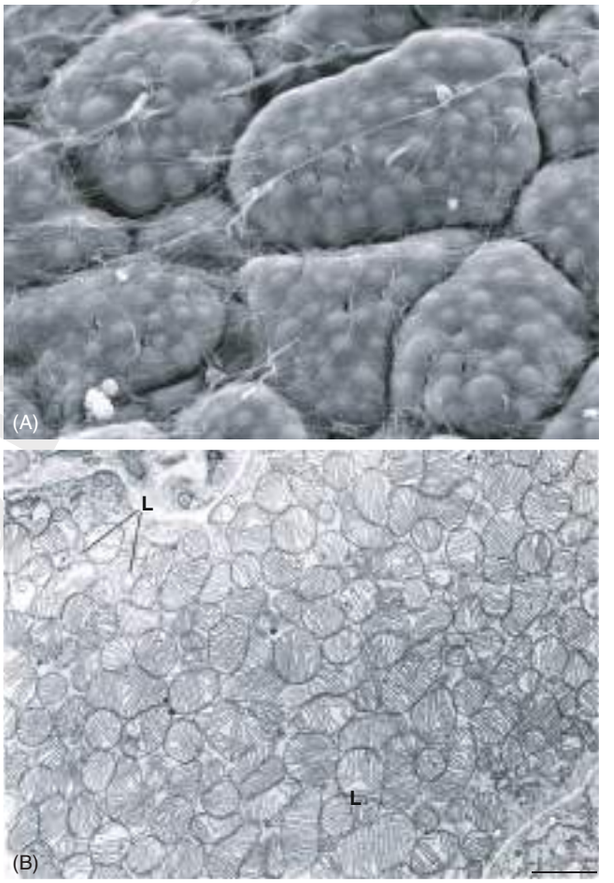

Figure 3 Scanning (A) and Transmission (B) Elecron microscopy of murine brown adipose tissue. Bar: in A, 6.0 μm; and in B, 1.0 μm. A adapted, with permission, from (167).

aspects both in the cytoplasm and nucleus (rich in euchromatin). Small lipid droplets and glycogen granules are often present (see also origin of adipocytes paragraph). Vascular and nerve cells in addition to their specific cellular ultrastructural features present topographic relationships, intrinsic to their anatomical organization, allowing even an easier identification by electron microscope (171).

WAT has a dense vascular and nerve supply, formed by unmyelinated noradrenergic nerve fibers and myelinated sensitive nerves (42,43,45,46,169). Many fibers are present in the perivascular areas but rare small noradrenergic fibers can also be found in the parenchyma (i.e., in contact with adipocytes) (332, 335).

Brown adipocyte

Brown adipocytes are polygonal cells smaller than white adipocytes (between half and one third). Their anatomy is quite different from that of white adipocytes. The nucleus is regularly round and usually located in the central part of the cell. Cytoplasmic lipids are organized in several small vacuoles (Fig. 2) (169,282,872). In addition, in brown adipocytes, the lipid vacuoles are limited by a membrane-free dense line containing the antilipolysis protein perilipin 1 (74, 356). Among the other lipid associated proteins perilipin 5 seems to be more specific of brown adipocytes and other highly oxidative tissues (462). Mitochondria are large, numerous, and packed with laminar cristae (Fig. 3) and contain a protein called UCP1 that is uniquely found in this cell type and widely considered marker of metabolically active brown adipocytes (110,306,735). Other organelles are similar to those described for white adipocytes, including the external lamina on the outer side of the plasma membrane. Dense granules similar to those found in endocrine cells are often visible in several areas of their cytoplasm (37, 169).

Brown adipose tissue

Brown adipocytes are organized to form brown adipose tissue (BAT) (Figs. 2 and 3). The brown color is due to the high density of mitochondria and vascular network. Each

brown adipocyte is in contact with three or more capillaries (624). In some instance, a single adipocyte can surround the whole circumference of the capillary wall with its cytoplasmic projections outlining the paramount importance of brown adipocytes-vessels functional relationships (154, 169). As a matter of fact, the capillary network density of BAT is five to six times that found in WAT (624). BAT is also highly innervated (Fig. 4A). Both myelinated and unmyelinated nerves are present in BAT (47, 111, 229, 332). Parenchymal nerve fibers run among adipocytes and confocal and electron microscopy reveal synaptoid contacts between parenchymal nerve varicosities and brown adipocytes (Fig. 4B). Synaptoid varicosities contain empty vesicles and a minority of dense core granules (624, 647). Immunohistochemistry data support their noradrenergic content although, in some specific anatomical sites (mediastinum) vesicular

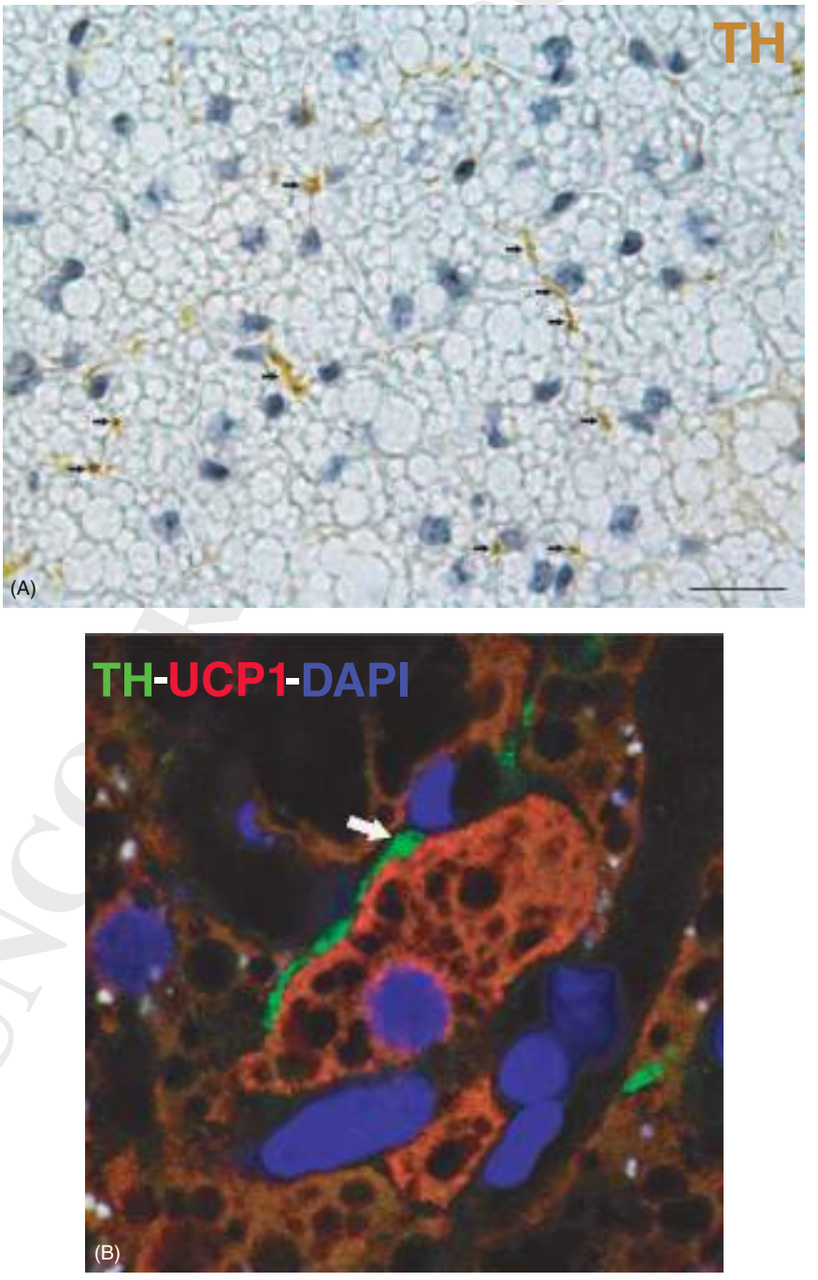

Figure 4 TH Immunohistochemistry (noradrenergic fibers) in murine (A) and human (B) brown adipose tissue. In B (confocal), a double staining UCP1/TH is shown. Bar: in A, 15 µm; and in B, 6.5 µm. B adapted, with permission, from (851).

acetylcholine transporter (VAChT) immunoreactivity support the coexistence of cholinergic parenchymal fibers (331).

Gross Anatomy

In anatomy, most of the available definitions of "organ" converge on the followings: a dissectible structure containing at least two different tissues responsible for different functions converging toward a single finalistic purpose. For example, the stomach, that is widely recognized as an organ, is composed by several tissues among which: epithelial glandular cells in the mucosa and muscular layers in its walls. Their functions are different: production of gastric juice (epithelium) and peristalsis (muscles), but both contribute to the final purpose of digestion.

The adipose organ is a dissectible structure

We showed that most of adipose tissues present in the body of mice of both genders and at different ages are contained into a large dissectible structure: the adipose organ (Fig. 5) (153-155, 157-159).

This large organ in lean animals weight about 15% to 20% of the total body weight and is composed by two tissues: WAT and BAT. A total of 60% to 70% of the organ is localized in the subcutaneous compartment where it forms two main depots at the root of the limbs. Thus, anterior and posterior subcutaneous depots (ASC and PSC, respectively) can be easy delineated. ASC is a complex depot located mainly in the dorsal part of the body in interscapular and subscapular area with cervical and axillary extensions. The subscapular, cervical, and axillary extension should be considered as intermuscular fat, thus not strictly belonging to the subcutaneous compartment. PSC is a band of fat starting in the dorsal area of the trunk and running in the inguinal region to join the contralateral symmetric part at pubic level where it is also continuous with gluteal fat (153-156, 158, 159).

Visceral depots are located in the trunk and surround aorta and its main branches: subclavian, carotid, intercostal, mesenteric, renal, and pelvic arteries. Omental fat is the only depot not in continuity with aorta or one of its branches.

We denoted a single visceral fat depot composed by perirenal, periovarian, parametrial, and perivesical parts as abdomino-pelvic (15, 159).

The adipose organ is mixed: Composed by two different tissues (WAT and BAT)

The relative amount of WAT and BAT of the organ is variable: depending on age, gender, strain, and by environmental and nutritional conditions (90, 177, 328, 364, 535, 537, 586, 614, 736, 793, 950).

Quantitative analyses showed that the adipose organ of adult female Sv129 and C57BL/J6 (B6) mice maintained at 28°C for 10 days have a relevant percentage of its parenchyma composed of multilocular adipocytes: that is, about 55% in Sv129 and about 30% in B6 mice (614, 950). About 90% and 50% of these multilocular adipocytes resulted UCP1 immunoreactive in Sv129 and B6 mice, respectively.

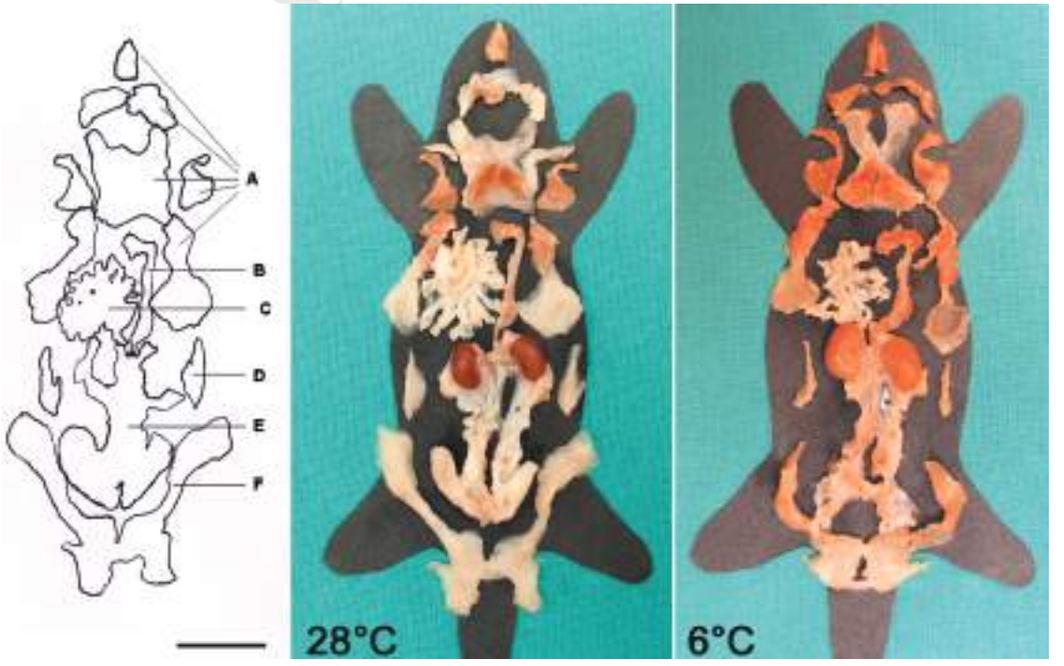

Figure 5 Murine adipose organ gross anatomy of warm and cold acclimated Sv129 adult mice. (A and F) Subcutaneous depots B to E visceral depots. Bar: 1 cm. Adapted, with permission, from (614).

Thus, parenchyma of the adipose organ of adult mice maintained at a temperature near thermoneutrality is mixed: composed by white (unilocular UCP1 negative), brown (multilocular UCP1 positive), and multilocular UCP1 negative adipocytes (ML/UCP1-). Of note, this last type of adipocyte has a range of morphology: from very similar to the brown to very similar to white adipocytes. The variable morphology visible at light microscopic level is due to variation in size of the cells and type of multilocularity. White adipocytes are larger than brown adipocytes and the largest ML/UCP1- cells approached the size of white adipocytes (Fig. 2). Their cytoplasmic lipids are organized to form a predominant central vacuole surrounded by several smaller vacuoles. We denoted this cell type as paucilocular cell (36). The smallest ML/UCP1- cells show a size similar to that of brown adipocytes and cytoplasmic lipids in form of regular small lipid vacuoles similar to those of brown adipocytes. A range of intermediate forms between the largest and smallest ML/UCP1- cells is always visible. Mitochondria of brown adipocytes exhibit a typical morphology: roundish, large, and packed with laminar cristae (181, 283, 623, 794). ML/UCP1- adipocytes show mitochondria with a range of morphology from the typical brown mitochondria to the nontypical morphology of white mitochondria (i.e., mitochondria of white adipocytes: elongated with few, randomly oriented cristae) (Figs. 1 and 3). Of note, the more the ML/UCP1- adipocytes approach the morphology of brown adipocytes, not only the morphology of the mitochondria approached that of typical brown mitochondria, but also their density in the cytoplasm increase (36, 428). Thus, all these morphologic and immunohistochemistry data support the idea that ML/UCP1- adipocytes are intermediate forms between white and brown adipocytes.

The distribution of brown adipocytes in the different areas of the adipose organ is similar in the two strains: in both the largest number of brown adipocytes was found in the ASC: about 80% in Sv129 mice and 65% in B6 mice. The high density of brown adipocytes in this region account for the brown color of several parts of ASC. The remaining brown adipocytes are located in the periaortic mediastinal fat and in the perirenal fat of both strains but also in perigonadal fat of Sv129 mice.

White adipocytes are mainly present in PSC and abdominal visceral depots: mesenteric, omental, perivesical, and retroperitoneal fat, thus the color of these areas is mainly white.

ML/UCP1-adipocytes with all the morphologic range are present in all transitional areas at the boundaries between WAT and BAT.

Vascular and nerve supply

The adipose organ is supplied by nerves and vessels usually reaching the organ in peduncles that can be easily isolated and dissected (169). We found vascular-nervous peduncles at the peripheral extremity of lateral wings of the interscapular fat of ASC, at the dorsal extremity of PSC, at the middle of the inguinal part of PSC. Isolated single nerves reach the organ in the ventral interscapular part of ASC and the lower part of the retroperitoneal fat. Many other diffuse small vascular-nervous peduncles and isolated nerves reach other areas of the organ.

Immunohistochemistry and ultrastructural studies of nerves in the adipose organ showed that it is provided with myelinated and unmyelinated nerves both in WAT and BAT areas (42, 43, 45-47, 296, 335, 340, 826). Myelinated are larger than unmyelinated nerves. Myelinated nerves are immunoreactive for Calcitonin-Gene-Related-Protein (CGRP) and Substance P (SP) both considered markers of sensitive nerves. The unmyelinated small nerves are immunoreactive for Tyroxine Hydrodylase (TH), considered a marker of noradrenergic nerves. In the vascular wall, Neuronal Peptide Y (NPY) in BAT areas have been identified (111). We found VAChT immunoreactivity (marker of parasympathetic fibers) in parenchymal nerves of BAT exclusively in mediastinal area (331).

Unitary finalistic purpose

Thus, the anatomical requirements to classify this structure as an organ seems to be fully satisfied but the cooperation between WAT and BAT to a common finalist role remains to be elucidated. In the next section, dynamic data of the organ (remodeling) offer an explanation for this functional aspect.

Development of WAT and BAT

The adipose organ is mixed, but many areas of ASC are formed by pure BAT and some areas of visceral depots, such as epididymal fat, are composed by pure WAT.

Most of the historical work on the development of BAT and WAT refer to these two anatomical sites to describe the developmental aspects of these two different tissues.

WAT

The murine fetal epidydimal WAT anlage is formed by a classic loose mesenchymal tissue without any specific characteristics. At postnatal days 4 to 6 the morphology changes into a very characteristic tissue formed by vasculo-adipocytic islets well delimited by fibroblast-like cells forming a clear boundary from the rest of the tissue that remain loose and poorly differentiated (Fig. 6) (305, 916). Instead, inside the islets large capillaries are surrounded by numerous pericytes and white adipocyte precursors at various stages of differentiation. White precursors are never found outside the islets strongly suggesting that the environment inside the islets is necessary for the precursors development. Apart from the cellular composition aforementioned described, one of the main difference between the inside and outside interstitial space is in the matrix composition: the collagen fibrils density is very high inside and loose outside.

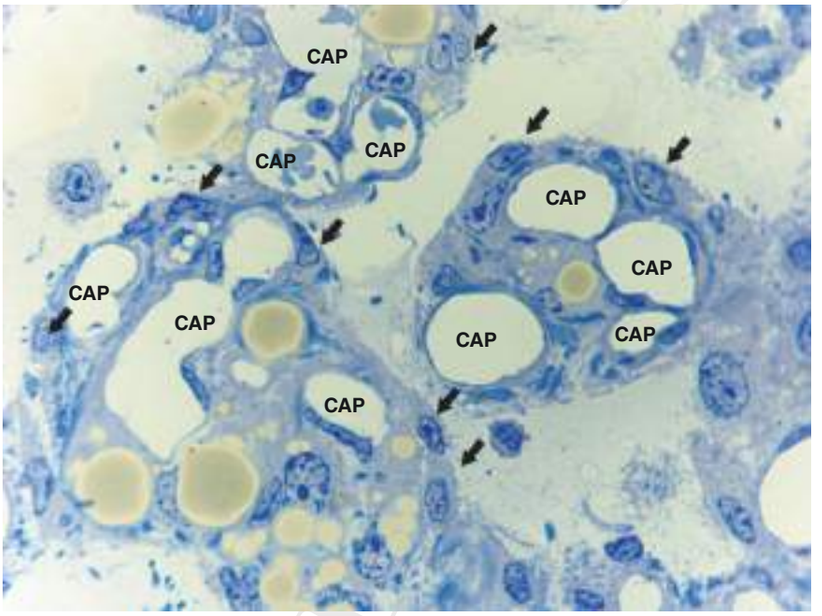

Figure 6 Vasculo-adipocytic islets of epididymal white adipose tissue from a newborn rat. Light microscopy. Bar: 7.0 µm. Adapted, with permission, from (169).

Thus, the islets result formed by a dense network of large capillaries rich in pericytes and immersed in a dense collagen stroma in which several steps of adipocyte development can be observed (Fig. 6). Together with these cell types, mast cells and macrophages are often present. Adipocyte precursor morphology is well established after many years of both *in vivo* (171, 622, 848) and *in vitro* studies (171, 172, 629, 847, 939). At early stages of differentiation, poorly differentiated cells, always in tight connection with capillary wall and surrounded by a distinct external lamina show high nucleus/cytoplasm ratio, few nontypical elongated mitochondria, glycogen granules, and few lipid droplets (Fig. 7A). Usually lipid droplets tend to coalesce quickly and most of the precursors assume a unilocular aspect with crescent-shaped nucleus even at early stage of differentiation when the cell is still small (5-10 µm in diameter) (Fig. 7B).

These white precursors show nuclear immunoreactivity for pRb (381) (a tumor suppressor protein playing also an important role in cell cycle and differentiation) (385, 528), -C/EBPα,-C/EBPβ, and PPARγ2 proteins and cytoplasmic immunoreactivity for S-100b (a multifunctional Ca2+binding protein) (173, 916).

The main morphologic aspect of development from early unilocular precursors and mature white adipocytes is the progressive enlargement of lipid droplet until the size typical for the specific fat depot: usually smaller in visceral than in subcutaneous fat (169, 275). This lipid droplet enlargement is accompanied by a progressive reduction in thickness of the cytoplasmic rim with apparent reduction in number and size of mitochondria (169).

Early precursors are very similar to pericytes (171, 622, 848, 916). In murine pericytes, lipid droplets are not found, but glycogen particles are often present. Pericytes are characteristically included in doubling of the basal membrane of capillaries. Some pericytes show an "extruding" morphology: that is, part of the cell included in the doubling of the capillary basal membrane and part of the cell abutting toward the interstitial space (153, 169, 916). These pericytes usually show cytoplasmic glycogen particles and could represent early stages of preadipocytes detaching from the capillary wall. Preadipocytes, at different developmental stages, are usually in contact with capillary walls with those less differentiated usually closest to the capillary wall. Of note, rare endothelial cells are in part in pericytic position (endothelial-pericytic cells) (169, 916).

Pericytes and endothelial cells are joined by gap junctions in the cytoplasmic projections apparently linking these cells (916). The physiologic significance of these joining projections is unknown, but can be found also in models of adipogenesis from tissue explants (916). Some endothelial cells containing glycogen clusters similar to those of pericytes are often found in developing epididymal fat at this stage (916).

BAT

In mice and rats, the first fat anlage, visible by light microscopy, appears around the 15th gestational day in the subscapular area of murine fetuses (169,644,834). In this area, dorsal skeletal muscles border a loose mesenchymal tissue characterized by the presence of a network of capillaries. Most

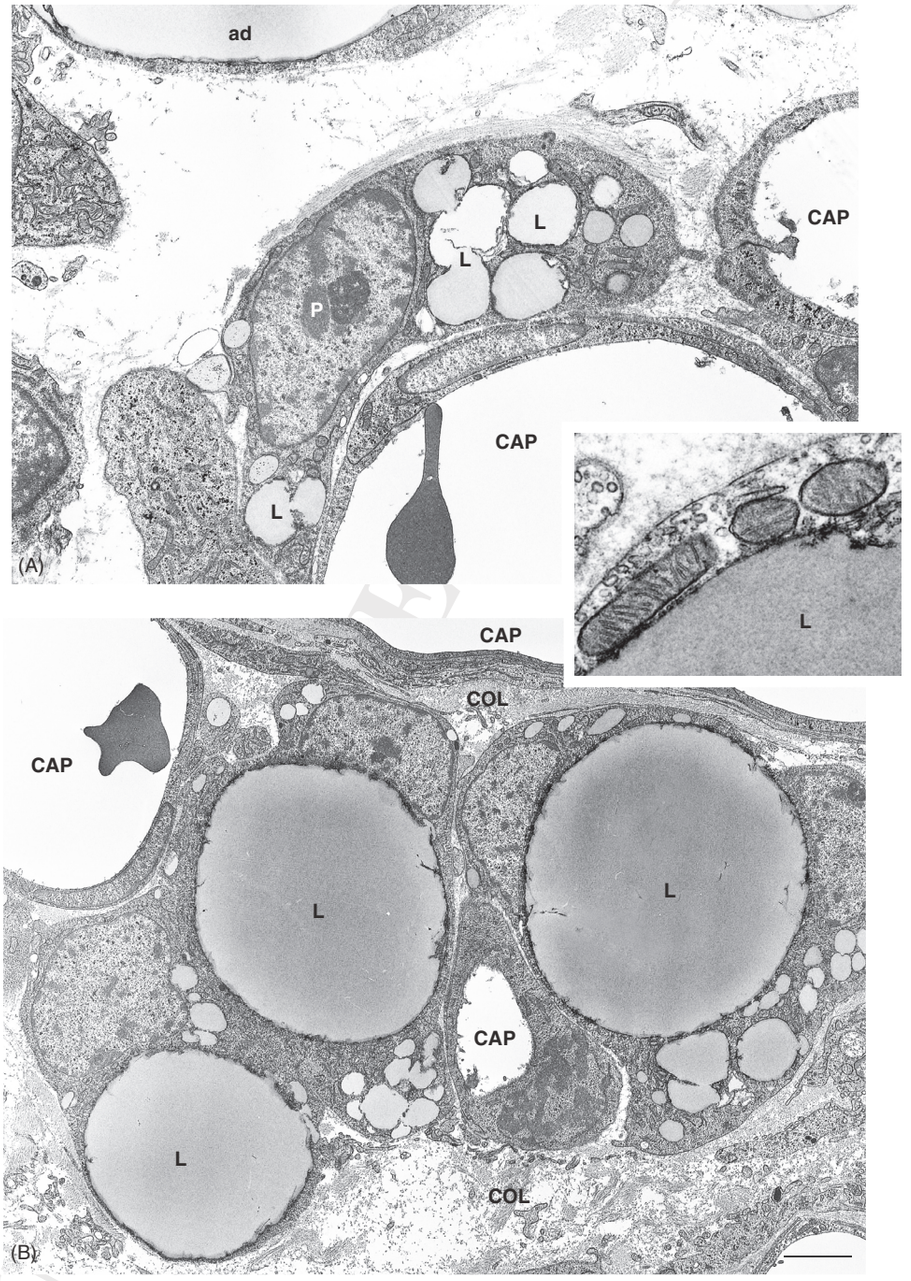

Figure 7 Transmission electron microscopy of vasculo-adipocytic islets shown in Figure 7. Bar: in A, 1.5 μm; and in B, 3.0 μm, in inset 0.5 μm.

of them contain also well-developed pericytes lining the capillary wall. A few interstitial cells are also present. Thus, at this very early stage of BAT anlage development three cell types are recognizable: endothelial cells, pericytes, and interstitial cells (Fig. 8A). At the 18th to 19th gestational day, the anatomy of the anlage is strikingly changed mainly because the loose tissue is substituted by a parenchymal-like tissue, filling up the meshes delimited by the capillary network (Fig. 8B). The cells forming this parenchymal-like tissue, often in mitosis, have the classic ultrastructure of brown precursors, with numerous large pretypical mitochondria (similar to those of mature brown adipocytes and often with large dense matrix granules) dispersed in a ribosome and polyribosome-rich cytoplasm (characteristic of poorly developed cells) (281). Large dense matrix granules of pretypical mitochondria have been suggested to be involved in cristae formation (37). 20-30% of

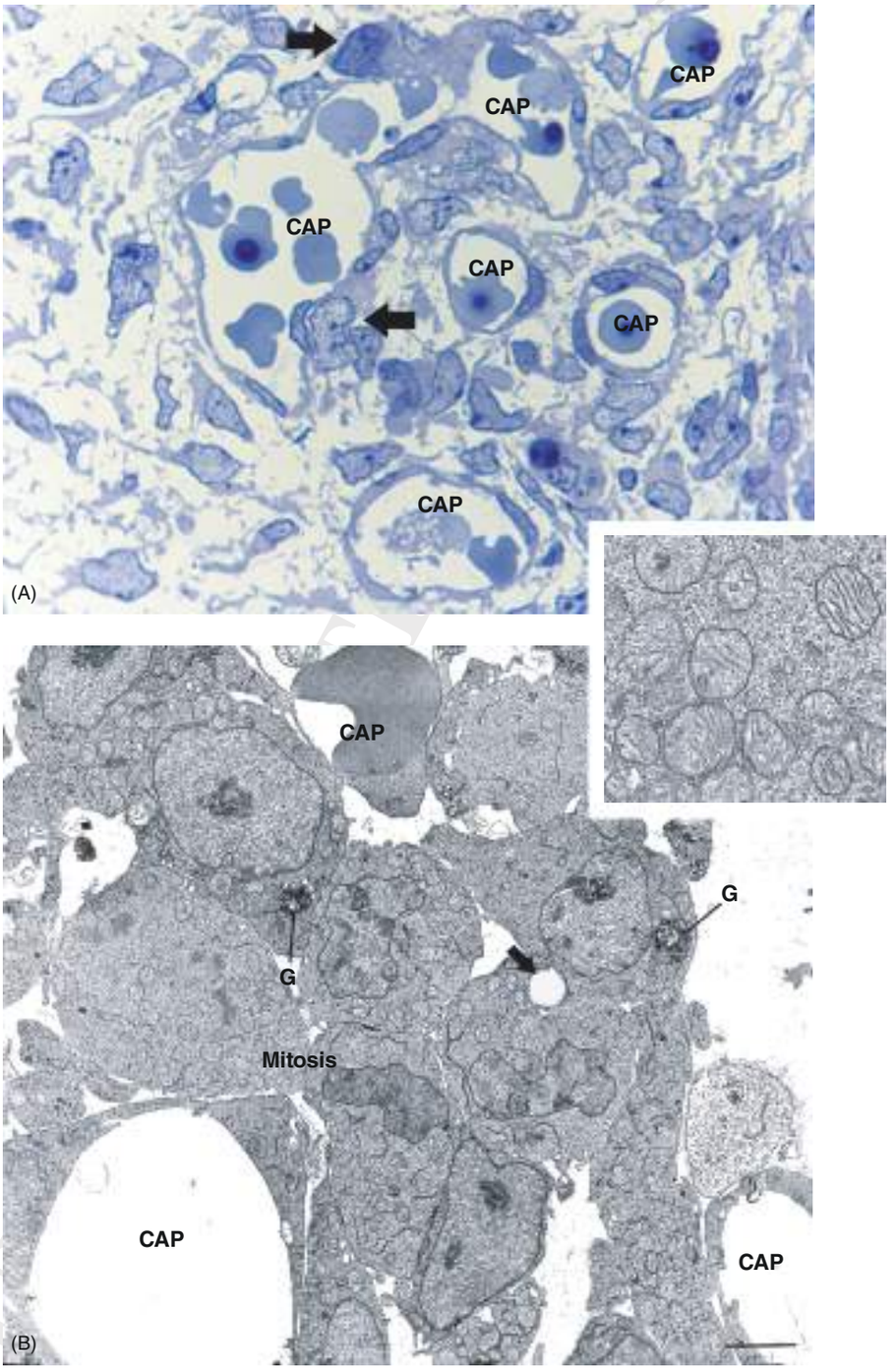

Figure 8 Rat brown adipose tissue anlage at E16 (A, light microscopy) and E18 (B, electron microscopy). Inset: enlargement of electron microscopy showing pretypical mitochondria. Bar: in A, 6 μm; and in B, 2 μm, in inset 0.6 μm.

these brown precursors show roundish clusters of glycogen and small lipid droplets, indicating adipose differentiation (153, 357, 358, 848) (Fig. 8B).

Immunohistochemistry revealed that brown precursors at this stage of development stain positive for PPARγ, C-EBPα, and β proteins in their nuclei and for UCP1 in their cytoplasm (916). At this developmental stage, brown precursors are not immunoreactive for Retinoblastoma (pRb) (381) or S-100b protein (35, 169, 250), proteins that are hallmarks of early white preadipocytes (see the preceding text) (173, 381, 585).

Many pericytes have an ultrastructure very similar to that of brown precursors, including pretypical mitochondria, and shared with them the nuclear immunoreactivity for the aforementioned transcription factors known to be typical of brown precursors from *in vitro* studies (180, 423, 916). Immunogold staining revealed the presence of UCP1 protein in the mitochondria of brown precursors and pericytes (916). Rare endothelial cells (1%-3%) show large mitochondria similar to those of pericytes and brown precursors (180).

By fetal days 20 to 21, just before birth, the morphology of brown precursors progressively approaches that typical of mature brown adipocytes (169).

Thus, morphology of white precursors is quite different from that of brown precursors and the main differences are in mitochondria (pretypical in brown, not typical in white) and in the lipid droplets (small and numerous in brown and unique in white) reflecting the morphologic differences of adult cells. Furthermore, immunohistochemistry revealed similarities in immunoreactivity for some transcription factors (C/EBPβ,α and PPARγ) in line with *in vitro* studies of adipogenesis (761) and differences for nuclear (pRb), cytoplasmic (S-100b), and mitochondrial (UCP1) proteins (35, 173, 381, 916). In both cases, preadipocytes are easily recognized by their specific features that make them distinct from other cell types (fibroblasts, macrophages, mast cells, lymphocytes, and granulocytes) that can be found in fat during development. The specific morphologic features are: a distinct external lamina surrounding a poorly differentiated cell with early signs of adipocyte development (37, 171, 357, 622, 848).

These data, in line with many other studies (368, 404, 505, 506, 508, 814), strongly point to the vascular wall as the site of origin for both white and brown adipocyte precursors.

Biogenesis of adipocytes

Old and recent studies seem to converge on the idea that the vascular wall of adipose tissue capillaries could represent the structure from which the committed adipocyte precursor both during adipose organ ontogenesis and in the adult life arise (57, 171, 890). Only two cell types form the wall of capillaries: endothelial cells and pericytes. Both cells can show morphologic evidence of some aspects of adipocytic differentiation (external lamina, glycogen clusters, pre-typical mitochondria), but immunoreactivity for markers of adipocytic commitments are found in pericytes (204, 505-508) and not in endothelial cells, although some rare endothelial cells can contain morphologic aspects (glycogen clusters, pretypical mitochondria) (180, 916) or proteins such as Zfp423 (369) (see also Fig. 9 suggesting a possible role of endothelial cells for both white and brown adipocyte precursors).

Lineage tracing is a potent technique to follow the developmental destiny of a specific cell type and it is based on the expression of reporter genes driven by the activation of gene promoters that can not only be cell specific (if a gene is

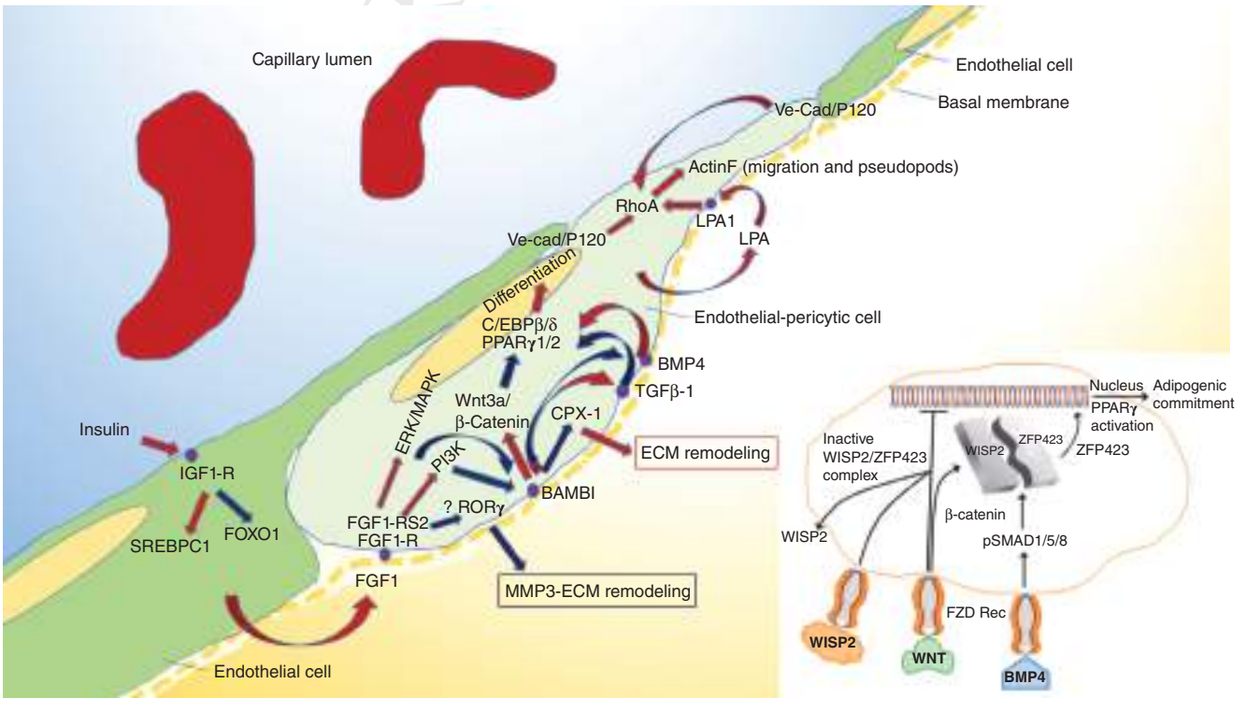

Figure 9 Scheme sho a hypothesis of molecular signaling inducing endothelial-pericyte-preadipocyte conversion. Inset adapted, with permission, from (378).

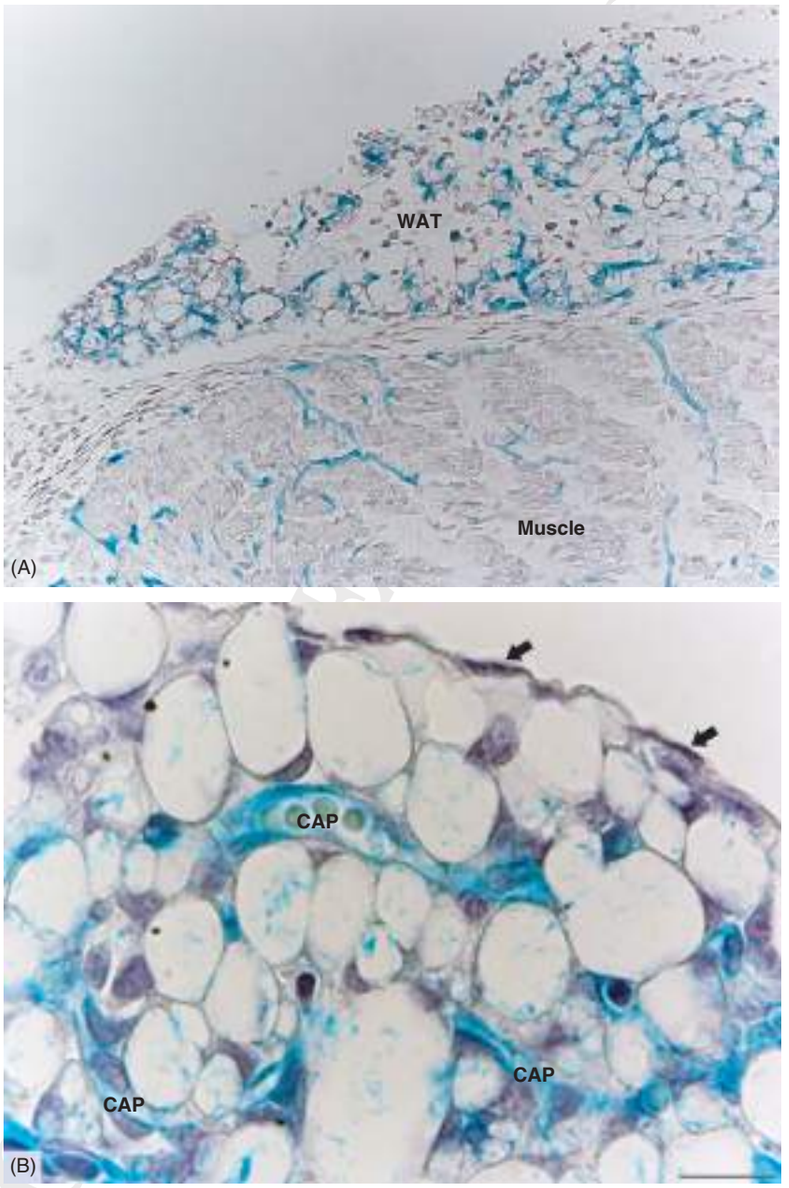

Figure 10 Inguinal adipose tissue from a Ve-Cad/Cre/R26R mouse showing lineage-tracing evidence of endothelial origin of adipocytes. Bar: in A, 330 μm; and in B, 15 μm.

expressed uniquely in that cell type), but also temporally specific (if a gene is expressed uniquely during a specific period of time) (854, 911).

We used VE-Chaderin-Cre/R26R mice that express the reporter gene (β-galactosidase) only in endothelial cells of all tissues in the body. In the original work Alva et al. (12) demonstrated that in these mice, none of the parenchymal cells of the organs studied (heart, kidney, brain, lung, intestine, retina, uterus, tongue, ovary, testis, salivary gland, mammary gland, adrenal, skeletal muscle, sclera, and choroids, and mesentery) expressed the reporter gene, with the exception of hematopoietic bone marrow stem cells (1021). In our experiments, both white and brown adipocytes of VE-Cadherin-Cre/R26R mice resulted marked by the reporter gene suggesting an endothelial origin of adipocytes in line with the morphologic data described in previous paragraph (Fig. 10A and B) (305, 916).

In line with our data, Shan et al. identified aP2-expressing adipocyte progenitors in SVF and endothelial cells of both WAT and BAT (814). Furthermore, in a recent study, perilipin+/adiponectin+preadipocytes were found to emerge at embryonic day 16.5 in inguinal WAT and proliferated to form clusters strongly interacting with growing adipose vasculature: that is, endothelial–specific *Vegfr2* depletion induced

vascular disruption with interruption of *in vivo* adipogenesis (404).

Capillary networks and single cell suspensions from microvessels of human fat explants give rise to well characterized adipocyte progenitors able to develop into mature adipocytes in a cell-autonomous manner (591).

In line with these data, isolated mature human adipocytes dedifferentiate into endothelial-like cells (125, 694, 697) and endothelial cells can be converted into mesenchymal stem cells which can differentiate into adipocytes, chondrocytes, and osteoblasts (581).

The endothelial origin would imply endothelial-mesenchymal transition (EndMT) that is linked to BMP/TGFβ signaling (554, 1007) and absence of the downstream effector myocardin-related transcription factor A seems to induce commitment of progenitors to adipogenesis (578).

Following the observation that interstitial cells, distinct from muscle satellite cells, in muscles have efficient adipogenic potential both *in vivo* and *in vitro* (432, 928), Granneman and colleagues showed that similar cells in abdominal visceral fat have a bipotential possibility to develop into white and brown adipocytes under appropriate stimuli *in vivo* (507).

This cell type has been described as stellate interstitial cells (PGFRα+, CD34+, CD24-, IB4-, and PDGFRβ-) provided with multiple thin cytoplasmic processes of about 50 μm in length located in close proximity to stromal cells, adipocytes, and capillaries in developed WAT. Similar CD34+ cells were described in human and murine subcutaneous fat (573). Their role as brown adipocyte precursors in WAT have been supported by experiments showing that suppression of the PDGFRβ+ population of stromal cells give rise to a compensatory proliferation of PDGRFβα+ cells ending in browning of WAT (214).

Recent data from human patients as well as data from murine experiments seem to support the bone marrow origin of at least a subpopulation of fat adipocytes (205, 323, 772) thus, a link between the hematopoietic multipotent population and endothelial cells with the potential ability to give rise to both white and brown preadipocytes and adipocytes cannot be excluded.

Vascular endothelium and pericytes seems therefore maintain the ability to differentiate into adipocytes, but other experimental data suggest that mesothelial cells can be the progenitors of visceral adipocytes (133), and other data seem to deny any role for endothelial cells (58) thus, the final picture of the origin question still offer the opportunity for future studies.

Molecular Mechanisms

White Adipocytes

Differentiation

Development of a new adipocyte is due to adipogenic induction of adipose stem cells and subsequent activation of differentiation signaling. Studies of white adipogenesis *in vivo* and *in vitro* have described a complex differentiation signaling. This transcriptional cascade involves the master regulator peroxisome proliferator-activated receptor γ (PPARγ), with its isoforms 1 and 2 and its heterodimeric partner RXR (retinoid × receptor), and three members of the CCAAT/enhancer-binding protein (C/EBP) family—C/EBPδ, C/EBPβ, and C/EBPα—that are activated sequentially (276, 421, 509, 757, 908). A recent paper elegantly confirmed the important role of PPARγ for both WAT and BAT differentiation in all anatomical sites using adiponectin-fat-specific knockout mice (955), but accurate comparison between *in vivo* and *in vitro* molecular mechanisms suggests peculiar aspect for *in vitro* adipogenesis (150).

C/EBPβ and C/EBPδ are induced transiently in the early steps and are considered to play key roles during the initiation of the adipogenic program. The two C/EBPs acts in cooperation and mice lacking both C/EBPβ and C/EBPδ have a severe lipoatrophic phenotype (889). C/EBPβ and C/EBPδ activation induces C/EBPα and PPARγ expression that are responsible for terminal adipocyte differentiation (510). In this complex network, the homeobox gene Hoxc8 suppresses C/EBPβ and this activity is very important to induce the white phenotype (327).

Other C/EBP isoforms such as CHOP (transcription factor homologous to CCAAT-enhancer binding protein) and C/EBPγ, seems to suppress adipogenesis, perhaps trough heterodimerization or inactivation of C/EBPβ (215).

Among other transcription factors required for adipocyte differentiation cAMP regulatory element-binding protein (CREB) seems to play an important role (731) and the E3 ubiquitin ligase murine double minute 2 (Mdm2) seems to be also involved in adipogenesis by promoting cAMP-mediated transcriptional activation of CREB and induction of C/EBPδ expression by facilitating the recruitment of CREB-regulated transcription coactivator (Crtc2) to a cAMP-response element (CRE) in the promoter of c/ebpδ (376).

In addition to C/EBPs factors, Krupper-like factors (KLFs) have been shown to activate at least one of the two PPARγ promoters even if the relative roles of PPARγ1 and PPARγ2 remain to be elucidated (758).

KLF 4, 5, 6, 9 ,and 15 have been showed to promote adipose differentiation with different mechanisms but KLF2, 3, and 7 are antiadipogenetic factors (32, 603, 655, 836). It has been proposed that appropriate exchange of these factors occur during adipogenesis (655). Many other factors are claimed to have a positive or negative role in this complex network of molecules interacting during adipogenesis are shown in Table 1 (544, 702, 758, 859).

Determination

Determination of stem cells toward adipose lineage is due to extracellular signaling acting on adipose stem cells upstream to the differentiative PPARγ-CEBPs transcriptional cascade described above. The nature of adipose stem cell *in vivo* is debated but the cell population of vascular walls of adipose

Table 1

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Transcription factors	
BMAL1	(Brain and muscle Arnt-Like protein-1, component of the molecular clock) Induces the expression of several factors involved in lipogenesis with circadian rhythm (452, 827).
EBF1, 2, and 3	(Early β-cell factor 1, 2, and 3). Control of genes of terminal differentiation. Direct action on PPARγ1 and C/EBPα promoter (7, 429).
EPAS1	(Endothelial-PAS superfamily, also known as hypoxia-inducible factor 2alpha). Promotes adipogenesis trough glucose uptake and lipid synthesis (828).
GATA2 and 3	Repress adipogenesis inhibiting PPARγ2 and C/EBPs (905, 906).
KROX20	(Early growth response protein-2). Promotes C/EBPβ (64, 141).
LKRα and β	(Liver X receptors). Their role is controversial (326, 417, 765, 812).
SREBP1c/ADD1	Induces PPARγ. Promotes insulin-mediated lipid synthesis (458, 907).
STAT5a	(Signal transducer and activator of transcription 5). Binding to subunits of the pyruvate dehydrogenase complex (PDC) that converts pyruvate to acetyl-CoA. PDC may modulate STAT5's ability to regulate gene expression by controlling histone or STAT5 acetylation (293, 621, 732).
Transcriptional, translational cofactors, and enzymes	
CBP	(CREB-binding protein) histone acetyltransferase (HAT) modifies chromatin directly promoting adipogenesis (989).
COUP-TFII	The orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (or Nr2f2) repressor of adipogenesis with direct action at the C/EBPα promoter (983).
ETO/MTG8	Corepressor acting on C/EBPβ antiadipogenetic action (745).
Fbxw7	(Human CDC4) is the substrate recognition component of a specific SCF ubiquitin ligase that targets for degradation C/EBPα (49).
HDACs/Sirtuins	(Histone deacetylates)/(HDAC activity): antiadipogenetic action (690, 998).
HRASLS3	(Phospholipase A2 superfamily). Target of PPARγ/RXR heterodimers. Induces adipogenesis (416).
IRFs	(Interferon regulatory factors). Repressors of adipogenesis (257).
NCoR/SMRT	(Nuclear receptor corepressor)/(Silencing mediator of retinoid and thyroid hormone receptors) antiadipogenetic action (1001).
p160 family	Scaffold to recruit chromatin modifier and HAT (543).
p300	HAT modifies chromatin directly promoting adipogenesis (884).
PLZF	Member of the BTB/POZ-ZF [Broad complex, Tramtrack, Bric à brac (BTB) or poxvirus and zinc finger (POZ)-zinc finger] protein family. Repressor of adipogenesis by recruiting nuclear receptor co-repressors (N-CoRs) and histone deacetylases (HDACs) (587).
SRF	(Serum response factor) a MADS-box transcription factor repressor of adipogenesis (587).
Stk40	(serine/threonine kinase 40) repressor of translational control of C/EBPβ and δ (1002).
SWI/SNF complexes	(ATP-dependent chromatin remodeling proteins) bind to and serve as co-activators of many nuclear receptors, including estrogen, glucocorticoid, retinoic acid receptor families, and PPARγ (233).
TAF8	(TATA-binding protein-associated factor-8, basal promoter binding factor) promote adipogenesis (363). Unclear the transcription factor interactive.
TRAP220	(Or PBP PPAR-binding protein) binding partner of PPARγ promote adipogenesis (324).
Cell cycle-related proteins	
CDK4	Activates PPARγ without its phosphorylation (69). It promotes anabolism by blocking catabolic processes (541).
CDK6	(Cyclin D3-cyclin-dependent kinase-6) complex phosphorylate PPARγ increasing its transcriptional activity (784).
Cyclin D1	Repress PPARγ (309).
Cyclin G2	Regulates adipogenesis through PPAR gamma coactivation (5).
E2F1	Induces PPARγ transcription during clonal espansion (273).
E2F4	Represses PPARγ expression during terminal adipocyte differentiation (273).
Cell fate coactivators	
HIC5	Binding partner of PPARγ in colonic epithelium. Direct cell fate to epithelial phenotype and drastically drop during adipogenesis (252).
Lats2	(Large tumor suppressor kinase 2) is one of the core kinases of the Hippo pathway and its action ultimately inactivate TAZ and reduces cyclin D1 promoting adipogenesis (13).
TAZ	(Transcriptional coactivator with PDZ-binding motif) interact with PPARγ to inhibit adipogenesis and allowing osteogenesis (102, 387).
TIP1/3	(Tension-induced/inhibited protein-1/3. TIP1 recruits HAT activity to muscle-specific promoters (myogenesis), TIP3 recruits HAT activity to Pparγ promoter (adipogenesis) (426).

tissues is a widely accepted niche for adipocyte progenitors (see the biogenesis paragraph for details). Some data support the possibility that endothelial cells of capillaries could give rise to adipocyte precursors both in WATs and in BATs (368, 916). The anatomical structure and topographical organization of endothelial cells of capillaries in developing fat imply the need for dynamic cellular adaptation and extracellular remodeling for an eventual endothelial-preadipocyte conversion and development. Molecular signals implicated in adipose determination of stem cells could be in line with the endothelial hypothesis (Fig. 9).

1. MAPK (mitogen-activated protein kinase) family members have been studied for their implication in adipogenesis and it has been shown that ERK1 (extracellular signal-regulated kinase-1) is required in the proliferative phase, but need to be reduced by MAPK phosphatase-1 in the terminal differentiation phase to prevent its inhibitory effect on PPARγ (85, 778). Interestingly MAPK family members promote endothelial adaptations to extracellular signals allowing homing of mesenchymal stem cells to injured tissues (1015). The molecular mechanism allowing stem cell homing include the activation of MAPK family signaling and this activity could be related to the endothelial-mesenchymal transition necessary for preadipocyte development from endothelial cells. As a matter of fact, during murine WAT development endothelial cells with unusual morphology and rare endothelial-pericytic cells have been documented by electron microscopy both in developing murine and human fat anlage (169, 916) (Fig. 9).

2. Adipose stem cells produce lysophosphatidic acid (LPA) that has been shown to activate RhoA (RAS homolog gene family member A) (502) and RhoA is also activated in endothelial cells by P120-Catenin, a VE-Chaderin associated kinase (777). RhoA play a role in cell transformation (necessary for the transition from endothelium to endothelial-pericyte cell) and a role for Rho-A in stem cells commitment is well established (577).

3. Furthermore, the FGF1-BAMBI system claimed to be important for adipose determination could play a role in endothelial-pericyte-preadipocyte conversion. It has been shown that microvascular endothelial cells of adipose tissue produce FGF1 (419). FGF1 via an FGF receptor 1/fibroblast growth factor receptor substrate 2(FRS2) activates the MAPK pathway (important for cell remodeling necessary to endothelial-preadipocyte conversion, see the preceding text). FGF-1 signaling also induces inhibition of BAMBI (bone morphogenetic protein and activin membrane bound inhibitor) activity that is a potent modulator of factors influencing adipogenesis such as Wnt and TGF superfamily members (TGF-β and BMPs, see the succeeding text) (547). Thus, a combined action of LPA-FGF1-BMPs-MAPK could play a role in the endothelium-pericyte-preadipocyte conversion during development (Fig. 9).

   Together with the morphological transformation and adaptations during the endothelial-adipose conversion, it is highly probable that an extracellular matrix remodeling is also necessary. The importance of extracellular matrix is supported by data showing that proteases able to remodel extracellular matrix have positive and negative effects on adipogenesis (9, 92, 521, 522, 583) and it has been shown that leptin modulates extracellular matrix molecules and metalloproteinases (126) (Fig. 9). Furthermore, the well-known fetal vasculo-adipocytic islets that give rise to adipose lobules both in visceral and subcutaneous murine and human fat are delimited by polarized fibroblast-like cells that are probably responsible for the intra islets collagen-rich microenvironment necessary for adipogenesis (162, 169, 916). Interestingly, FGF-1 induces the inhibition of BAMBI and consequent activation of carboxypeptidase x-1 (CPX-1) that induces an extracellular matrix remodeling necessary for adipogenesis (461). RORγ play a negative role on adipogenesis through expression of its target gene matrix metalloproteinase 3 (MMP3) (584). Thus, it could be hypothesized its downregulated by FGF-1 activation.

4. The strong inhibitory stimulus to adipose commitment due to Wnt signaling downstream to FGF-1/BAMBI system could be removed by a combined action of this system and BMP proteins (305, 627).

Thus, ERK/MAPK signaling, LPA, and FGF1 could be key signals to activate the determination of adipose stem cell to preadipocyte, but which signal induce these pathways is not known. Insulin exert a potent effect on adipogenesis (175), probably trough insulin growth factor-1 (IGF1) receptor signaling because preadipocytes express more receptors for IGF-1 than for insulin (849). Insulin induces inactivation of FOXOs (forkhead box proteins) (inhibitors of adipogenesis) trough AKT (serine-threonine protein kinase) and activation of SREBC1 (activator of adipogenesis, see TAB I) through mTOR (mammalian target of rapamycin) (373, 617). Members of Bone Morphogenetic Proteins (BMPs, see also paragraph on endocrine functions) family have been suggested to play a key role in the commitment of adipocyte precursors and in particular BMP7 for brown adipogenesis (922) and BMP2 for white adipogenesis and BM4 for both white and brown adipogenesis (94, 264, 371, 372). Intrinsic production of BMP4 during preadipocytes differentiation would induce adipogenic commitment by the dissociation of an intracellular cytosolic complex formed by ZNF423 (zinc-finger-protein) and WISP2 (WNT1-inducible signaling pathway protein-2). ZNF423 is a transcriptional activator of PPARγ (367) found in early adipocyte precursors and adipose stem cells (368). Its role in adipocytes seems also to be important to maintain the white phenotype inhibiting the activity of Ebf2 and suppressing Prdm16 activation (818). The ZNF423/WISP2 complex

dissociation would allow ZNF423 nuclear entry and PPARγ activation (Fig. 9 inset).

Other BMP regulators should play an important role in adipogenesis and several potential inhibitors/activators have been found in adipose organ, such as activin (1006), follistatin (292), Dickkopf (51, 372), Kielin/chordin-like protein (523), and Dm/Gremlin (370, 909).

Another pathway that plays a role in adipogenesis is the ancient hedgehog signaling pathway. In rodent models, it seems that the inhibitory effect of hedgehog proteins act through the antiadipogenetic factor GATA2 (877), in human models data suggest that hedgehog does not interfere with induction but instead with maturation of human adipocytes (294). Recently, it has been reported that ciliary transduced hedgehog signaling regulated the expression of TIMP3, a secreted metalloproteinase inhibitor, that inhibited matrix metalloproteinase 14 to block intramuscular adipogenesis (471).

The transforming-growth factor β (TGFβ) superfamily members, including the above reported BMPs and myostatin, regulate the differentiation of several cell types including adipocytes (94).

TGFβ proteins activate SMAD-dependent and independent mechanisms and are inhibitory of adipose differentiation mainly through the effects of SMAD3 on C/EBPs (146, 147). Interestingly FAD-104 (factor for adipocyte differentiation 104) a positive regulator of adipogenesis (903), has been shown inhibitory properties on TGFβ in cancer cells (350).

Myostain action on adipogenesis is debated (22, 23, 285, 366, 724), but a recent quantitative study, using label-retaining wild-type and myostatin −/− mice, support its inhibitory effects on adipogenesis (518).

Notch is a transmembrane receptor that translocates to the nucleus after ligands-induced cleavage. In the nucleus, Notch binds to and activates RBP-jk transcription factor. This signaling is required for differentiation of 3T3-L1 preadipocytes (317) and its absence induces the inhibitor of differentiation Dlk1/PREF1 (764). In addition, direct promoting activity on PPARγ expression has been suggested (25, 813).

PREF1 (Preadipocyte factor 1 or Dlk1/FA1) is a molecular gatekeeper of adipogenesis that acts by preventing adipocyte differentiation. It is a transmembrane protein cleaved by TNFα-converting enzyme to generate a soluble form which interacts with fibronectin and activates integrin signaling though ERK/MAPK to inhibit adipocyte differentiation (414).

Bromodomains containing proteins seems to play an important role in chromatin regulation and transcriptional control of adipogenesis (232).

Using a cocolture method of adipocytes/preadipocytes, a proteomic study showed that several inhibitors/enhancer factors could be involved in the neo-adipogenesis phenomenon and Slc27a1(long-chain fatty acid transport protein 1), Vim (Vimentin), Cp (Ceruloplasmin), and Ecm1 (Extracellular matrix protein 1) secreted factors have been suggested as promoters of adipogenesis (130).

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Table 2

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Wnt	miR-8 (453), miR-120, miR-148a (823), miR-210 (822), miR-335, miR200 (822, 823).
MAPK/ERK	miR-375, (822, 852, 1010), miR-143 (269).
KLF	miR-146b (136), miR-448 (852).
PPARs-C/EBPs	miR-27a,b (524), miR-130a, miR-519d (565), miR-138, miR-31, miR-326, miR-155 (852) Insulin signaling miR26b (853, 978), miR-29, miR31, miR-93 (140), miR-103/107, miR-146b (6, 915), miR-143 (436), miR-320, miR-375 (852).
TGF/BMPs	miR-21, miR-199a (852).
CREB	miR-124, miR-132, miR-155 (852).
Cell cycle	let-7, miR-15a, miR17-92 (852, 958), miR-1908 (990)
Cell fate	let-7 (881, 963), Ret-7, miR-30, miR-204 (822, 852), miR-221/222 (845), miR-320 (377).

Many other miRs have been shown to be involved in adipogenesis, but their targets are still unknown. Proadipogenetic activities have been shown for miR-24, miR-107, miR-150, miR-200, miR-335, miR-378 (121, 270, 361, 579, 968, 1017). Antiadipogenetic properties have been shown for miR-326 (891).

Furthermore, the hormone leptin produced by adipocytes could also play a role in the preadipocytes recruitment (see paragraph of endocrine properties of adipose organ) in adult animals (see the succeeding paragraph on leptin).

MicroRNA MicroRNAs are a class of small noncoding single-stranded RNAs able to silencing mRNAs and posttranscriptional regulation of gene expression.

A growing body of evidence suggests positive or negative roles for several MicroRNA on critical steps in both aspects of adipogenesis (determination and differentiation), see Table 2 (6, 395, 822, 823, 958, 975). Interestingly, the obesity-associated gene Fto activity affects genes regulating adipogenesis (753, 896, 1014) and data support a functional link between FTO and miRNAs (754).

Brown adipocyte determination and differentiation

The determination and differentiation signaling for brown adipogenesis is less known and studied, but several important molecular signaling specific for brown adipogenesis have been identified.

Key transcriptional factors The key transcriptional factors for white adipogenesis C/EBPs-PPARγ are indispensable also for brown adipogenesis, and intense immunostaing for PPARγ was found in nuclei of fetal brown preadipocytes (916) in line with data showing its dramatic reduction in nuclei of brown adipocytes of adult mice (740). Importantly, C/EBPβ, with its complex network of regulators, including CREB, Hoxc8, Plac8, KSR1, and TRB3, plays a

predominant role, instead C/EBPα seems to be dispensable for brown adipogenesis (60, 355, 384, 430, 472, 527, 889).

As for white adipogenesis Wnt signaling plays an important role also for brown adipogenesis and a block of differentiation for brown preadipocytes have been shown (382).

An important role is played by the key activator of brown adipocytes: noradrenaline. *In vitro* and *in vivo* studies support the fact that noradrenaline acts on β1 adrenoceptors to induce the first steps of determination and differentiation on adipose stem cells probably localized in the vascular wall of fat microvasculature (36, 50, 100). However, mice lacking all β-adrenergic receptors develop a quite normal BAT anlage containing brown precursors with ultrastructural morphology very similar to that typical of wild type mice (169). These precursors develop into adipocytes with morphologic and gene signature more similar to white than brown fat (26, 723), suggesting that noradrenaline induces differentiation but not determination of adipose stem cells. The signaling downstream the β-adrenergic stimulus plays an important role for brown differentiation because the compensatory upregulation of RIα in mice lacking the PKA regulatory subunit RIIβ, induces a hypersensitivity of cAMP to PKA and consequent increase of brown phenotype (206). Furthermore, hyperexpression of Foxc2, a factor that controls the expression of RIα, in fat, also induces a brown phenotype (127), but its role has been questioned by experiments showing that forced expression of Foxc2 blocks the adipose conversion of 3T3L1 preadipocytes (218). However, a recent paper confirmed the importance of Foxc2 for the induction of a brown phenotype of adipose tissue (316).

Fetal adipose stem cells are induced to differentiation by insulin (934) and recent data support a role for two pathways involved in brown differentiation: activation of CREB via Ras-ERK1/2 and deactivation of FoxO1 via Akt. Both IRS-1 activated pathways combine to decrease necdin (nuclear protein inhibitor of E2F-mediated PPARγ 1 activation) levels and consequent brown gene expression (20, 88, 213, 346, 465, 921, 933).

Morphologic-molecular correlations Electron microscopy allows for the distinction between white and brown preadipocytes at very early stages of differentiation both during fetal development or during recruitment into the adult adipose organ (37, 158, 169, 622). The main morphologic differences consist in the quantitative and qualitative characteristics of mitochondria (see biogenesis of adipocytes paragraph). In line with these data, molecular signaling related to mitochondriogenesis and to the brown-specific UCP1-bearing mitochondriogenesis are distinctive features of brown adipogenesis. The proximal and distal promoters of UCP1 contain CREB (c-AMP response-element binding protein) binding sites, further outlining the importance of noradrenergic signaling for differentiation of brown preadipocytes (110, 382). Of note, UCP1 protein is detected by immunogold cytochemistry at very early steps of differentiation (916) and ectopic expression of UCP1 in WAT induces brown mitochondriogenesis (768) suggesting that the early events of differentiation include the activation of UCP1 promoter. The distal enhancer is necessary for brown adipogenesis and bears binding sites for TRs (Thyroid Receptors), PPARs and RXRs (Retinoid X Receptors). PPARα is specific for brown differentiation and PPARγ2 appears dispensable for brown adipogenesis (474), in line with data suggesting that PPARγ2 plays a dominant role for white adipogenesis (582, 1009), with the suppressive role of noradrenaline on PPARγ2 (525) and with quantitative data showing the predominance of PPARγ1 during brown adipocyte development (740).

At very early stages of fetal adipose organ development, immunohistochemistry showed the differential expression of two proteins: pRb (a tumor suppressor protein playing also an important role in cell cycle and differentiation) and S-100b (a multifunctional Ca^{2+}-binding protein) (35, 173, 381) only respectively in nuclei and cytoplasm of white precursors. Interestingly, suppressing the activity of pRb induces brown phenotype in developing preadipocytes and it is inhibited by β3AR agonists with consequent white to brown transdifferentiation of mature adipocytes (382). On the other hand, S100b is expressed by brown adipocytes during brown to white conversion (35) suggesting the existence of common pathways between differentiation and transdifferentiation.

Master regulators of brown differentiation PGC-1α (PPARγ coactivator 1α) plays a key role for the brown-specific mitochondriogenesis acting on NRF1 (nuclear respiratory factor1) and Tfam (mitochondrial transcription factor A) (384, 708). These factors are regulators of respiratory chain genes and of replication of mitochondrial genome respectively. Of note, PGC-1α is probably the main target of the inhibitory Wnt signaling (382) and is activated by the adrenergic/CREB induced SIRT3 (member of sirtuin family with protein deacetylase and ADP-ribosylase activity) and by NO (adrenergic-dependent gaseous regulator nitric oxide) (638).

Several other factors drive the brown-specific phenotype inhibiting other phenotypes. Among them PRDM16 (PR domain zinc finger protein 16) through the activity of EHTM1 (euchromatic histone-lysine N-methyltransferase1) and with the cooperation of interactive partner ZFP516 (zinc finger protein 516) plays a dominant role in the inhibition of muscle phenotype (653, 960). Its interaction with C/EBPβ is sufficient to induce brown adipogenesis from skin fibroblasts (441). EWS (Ewing sarcoma breck-point region1) and YBX1 (Y-box-binding protein 1) also inhibit the muscle phenotype and activate the expression of BMP7 that promotes brown genes (423).

EBF2 (early B-cell factor2) is a transcription factor expressed in embryonic brown preadipocytes and its activity is important to maintain the brown phenotype (960). Of note, ZFP423 is required to maintain the white phenotype suppressing EBF2 (817, 818).

A complex network of nuclear receptors coactivators and corepressors including the p-160 family, RIP-140, ZIP13, and

SRF are also likely to play a role in the differential phenotype between white and brown adipogenesis (313, 382, 758, 762).

MicroRNAs Several microRNAs play a role in brown adipogenesis regulating some of the above reported pathways.

miR-155 is an inhibitor of brown adipogenesis. It has been shown that there exists reciprocal negative regulation between miR-155 and C/EBPβ that integrate pro- and antiadipogenetic cues, including the autocrine activity of TGFβ1 (antiadipogenetic inducer of miR-155). When proadipogenetic stimuli prevail the inhibitory effect of C/EBPβ on miR-155 allows brown differentiation (139).

Another negative regulator of brown adipogenesis is miR27 that is down regulated by cold exposure. miR-27 inhibits the main actors of browning: PPARα, PRDM16, CREB, and indirectly PCC1α (879).

miR-196a suppresses the white-fat gene Hoxc8 (inhibitor of C/EBPβ, see the preceding text) allowing the brown phenotype of adipocyte precursors (600).

The cluster 193b-365 is induced by PRDM16 to inhibit the adipogenic repressor Runx1t1 (880).

miR-133 is a PRDM16 repressor and is downregulated by cold exposure allowing the brown phenotype of adipocyte precursors (530, 914, 996).

miR-455 activates AMPK (regulator of ATP production thought fatty acids oxidation) promoting the brown adipogenic program and mitochondrial biogenesis. Concomitantly, miR-455 also targets the adipogenic suppressors Runx1t1 and Necdin, initiating adipogenic differentiation (1008).

The cluster miR-106b-93 seems to inhibit brown differentiation acting on PPARα (975).

Interestingly, most of the above reported developmental browning mechanisms play also a role in adrenergic-induced browning of mature white adipocytes, further supporting similarities between developmental and transdifferentiation molecular mechanisms.

miR-26 family induces brown phenotype genes in HMADS cells (a well-defined model of human cell line derived from subcutaneous WAT) acting with inhibitory effects on the sheddase ADAM17 (447).

miR-378 controls specifically interscapular brown adipocytes expansion targeting Pde1b, a phosphodiesterase that catalyzes the turnover of cAMP and cGMP (673).

Adipose Organ Physiology

The main functions of this organ are thermogenesis and storage of fuel that can be released to the entire organism on demand. BAT is responsible for the first and WAT for the second purpose, but in case of special needs, such as prolonged cold exposure or chronic positive energy balance homeostatic plastic remodeling mechanisms are activated to satisfy these special needs: that is, during chronic cold exposure the BAT component increase (browning) and during chronic positive energy balance the WAT increase (whitening) allowing a high level of adaptability of the organ to satisfy these two important functions.

Thermogenesis

When the animal is exposed to a temperature below thermoneutrality (threshold temperature below which thermogenesis is induced) BAT, that is, all brown adipocytes contained in the adipose organ, either as compact tissue (interscapular, subscapular, deep cervical, and mediastinal) or as interspersed cells among white adipocytes (axillary, inguinal, perirenal, and perigonadal) produce heat (109, 397). If cold exposure is chronically prolonged (days or more), the browning phenomenon extend the heat production to a progressively larger number of brown adipocytes until a sufficient amount of thermogenesis is reached.

Activated BAT modify its morphology: lipid vacuoles become smaller and mitochondria assume a spherical shape and increase their number and cristae density (169, 397, 737, 967). Parenchymal nerve fibers increase their density (229) and jap junctions between brown adipocytes, responsible for their electric coupling, extend their areas (34). Thus, several morphologic signs mirror the functional activity of BAT and therefore its morphology strongly depend from the level of thermogenesis required by the organism. As a matter of fact, the BAT activity starts when the animal is exposed to a temperature below thermoneutrality and increase progressively in parallel with cold (397).

Molecular mechanisms

The molecular mechanism inducing heat production is essentially due to the activity of the mitochondrial uncoupling protein UCP1 that is activated and neo synthesized in brown adipocytes. When cold is sensed mainly by skin receptors (397) but also by adipocytes (995), nervous afferent stimuli reach the hypothalamus (probably ventromedial nucleus) and the sympathetic nervous system is activated to secrete noradrenaline in all areas of the organ reached by parenchymal nerve fibers (44, 110, 486). β3ARs of brown adipocytes are the main adrenergic Gs protein coupled receptors responsible for their thermogenic activity. Their activation induces a signaling cascade mediated by adenylyl cyclase activation and cAMP formation. cAMP activates protein kinase A (PKA), that trough phosphorylation of a series of target enzymes leads to the final functional effect. In particular, the phosphorylation of the transcription factor CREB (cAMP response element-binding protein) activates the gene expression of UCP1 (110, 114).

PKA also induces the activation of a second MAP kinase pathway, the p38 pathway (115, 194). p38 MAPK has been shown to be an important downstream target of the beta-adrenergic/cAMP/PKA signaling pathway in adipocytes, and one of the functional consequences of this cascade is stimulation of UCP1 gene expression in brown adipocytes. A

recent study also demonstrates that mitochondrial reactive oxygen species, which accumulate in stimulated brown fat cells, enhance UCP1-mediated respiration by promoting the sulfenylation of a cysteine residue in UCP1 itself (145). Furthermore, possible roles for mTORC1 (a nutrient sensor mechanistic target of rapamycin complex 1 which plays a key role in coordinating anabolic and catabolic metabolism at the cellular level) (33) and the liver kinase b1 (Lkb1) has been also suggested. Adipocyte-specific mTORC1 loss in mice completely blocks cold-induced BAT expansion and severely impairs mitochondrial biogenesis (487). Adipocyte-specific Lkb1 loss induced UCP1 activation trough C/EBPβ recruitment and activation of mTOR (815). β3ARs activation induces also lipolysis via two PKA mediated processes: activation of hormone-sensitive lipase (HSL) and phosphorylation (deactivation) of perilipin1. Lipolysis results in free fatty acids in the cell that are preferentially directed toward mitochondria where they serve as substrate for thermogenesis and likely are also involved in the regulation of UCP1 activity (110). The β-oxidation of fatty acids results in the pumping out from the mitochondrial matrix of the protons and consequent formation of mitochondrial membrane potential gradient. The presence of the mitochondrial carrier protein UCP1 explains the thermogenic function of the cell: uncoupling the β-oxidation of fatty acids from ATP synthesis, all the heat derived as an inevitable secondary effect of the biochemical reaction serve as principal final product of the cell activity: thermogenesis (89, 735). Other molecular mechanisms could be implied in BAT activation and recently the lipid 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) has been identified as a stimulator of BAT activity (548). Because mitochondria are numerous, large and packed with cristae the final result is that the heat produced is relevant for a physiologic role. Furthermore, the multilocular organization of lipid droplets into brown adipocytes allows a rapid disposal of a large amount of free fatty acids ready for oxidation in mitochondria. It has been calculated that the heat produced by a stimulated brown adipocyte is about 300 times that in average produced by other cell types for their routine biochemical work (628). Brown adipocytes are electrically coupled by jap junctions (802) that increase their size during cold exposure (34). Furthermore, during chronic cold exposure peripheral parenchymal noradrenergic nerve fibers density increase (611) probably with branching mechanism guided by endocrine and paracrine factors (see the succeeding text), and a progressively higher number of white adipocytes is reached by noradrenaline. Recent papers showed that the histone deacetylase 3 (HDC3) and the nuclear factor I-A (NFIA) are necessary to activate BAT enhancers to ensure thermogenic disposition (265, 400).

White adipocytes are provided with β3ARs (84, 224) able to respond to noradrenaline with a white-to-brown conversion to increase the total number of thermogenic brown adipocytes (see the succeeding text). Mammals survive in environments from about +50°C to about −60°C, thus the internal temperature 37°C (humans) is closer to the highest than to lowest environmental temperatures denoting the need for important thermogenic equipment. The adipose organ responds to the thermogenic request with BAT activation and WAT browning.

The Harlequin phenomenon

Acute exposure to cold induces also the so-called Harlequin phenomenon in the BAT areas of adipose organ (170). We showed that acute cold exposure or β3AR agonist administration induces an intense UCP1 immunostaining or UCP1 mRNA expression in situ of some brown adipocytes in BAT among other that remain completely negative or only weakly immunoreactive, thus the histology results in a tissue formed by polygonal shaped cells differently colored reminding the Harlequin mask (Fig. 11). Interestingly, morphometric analysis of immunogold-stained thin sections showed that UCP1-gold particle density in mitochondria is different among neighboring brown adipocytes with mitochondria of the same size and cristae density. After chronic cold exposure or β3AR agonist administration the Harlequin attenuates and UCP1 immunostaining result less intense per single cell. Serial sections immunostained with UCP1 and the heat-shock protein (HSP) HO1 showed an intense nuclear positivity for the HSP in the intensely UCP1 stained adipocytes, suggesting that acutely activated brown adipocytes protect themselves from heat-shock promoting neo synthesis or activation of proteins able to inactivate the thermogenic system (170). The necessary thermogenesis would therefore be guarantee by the alternate activation of brown adipocytes. Chronically activate BAT seems to reduce the amount of heat per cell with a more widespread activation of a large number of adipocytes. This increase could be explained by the branching of parenchymal nerve fibers and expanded activity of coupling jap junctions. Cold exposure induces increased number of brown adipocytes (605, 950) and proliferation of PDGFRα immunoreactive brown precursors in interscapular brown fat by the activation of β1ARs (506). Thus, the thermogenic capacity of adipose organ in cold exposed animals is also due to development of new brown adipocytes.

WAT browning

Chronic cold exposure requires a progressive increase in number of thermogenic brown adipocytes, thus the white areas of adipose organ turn into brown: this phenomenon is also known as browning of adipose organ and is visually evident in dissected organs of cold acclimated mice (Fig. 5) (153, 155, 157, 159, 166, 220).

Browned areas contain an increased amount of BAT-like tissue that is present in small quantities also in warm acclimated mice (see the aforementioned paragraph on gross anatomy) (614, 950).

The first paper dealing with brown adipocytes in WAT demonstrated a substantial increase of brown adipocytes in the parametrial WAT of cold exposed mice (1000). Then a series of papers showed the remodeling of the tissue toward a BAT-like tissue and appearance of multilocular adipocytes (without

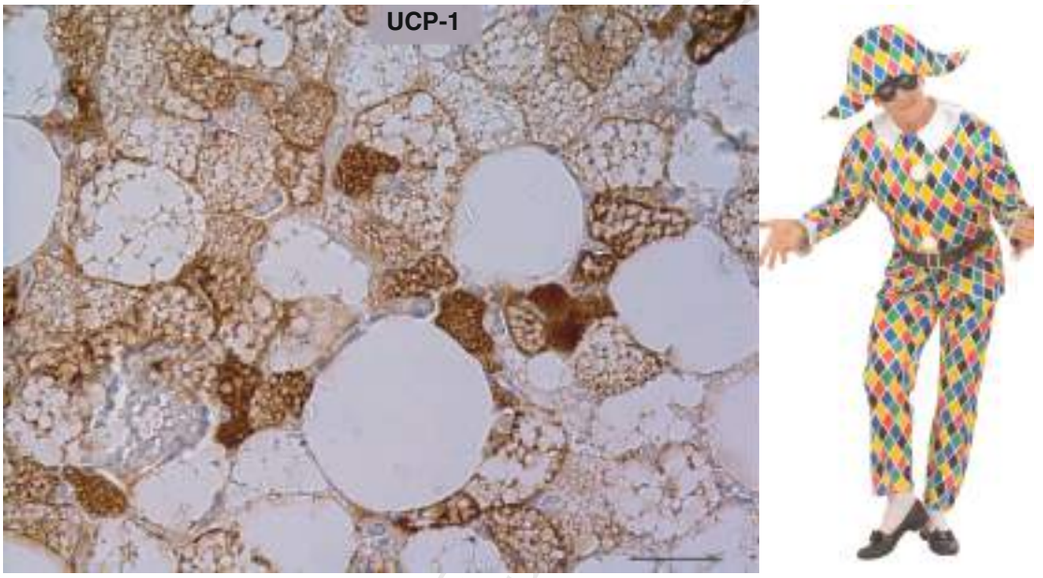

Figure 11 UCP1 immunohistochemistry of acutely activated brown adipose tissue show the heterogeneous immunoreactivity (Harlequin phenomenon). Bar: 35 µm.

UCP1) after cold exposure in epipdydimal WAT of rats (535, 536) and in 1991 a reversible browning of inguinal fat of cold exposed mice was shown and the tissue was described as convertible adipose tissue (533, 534).

In 1992, Cousin et al. (202) showed the progressive molecular and morphologic transformation of periovarian white fat of cold exposed rats. In this paper, many molecular and morphologic steps of white-brown conversion were described and the presence of UCP1-immunogold particles in mitochondria of unilocular adipocytes of cold exposed animals were described for the first time. Furthermore, in that paper, the presence of jap junctions (typical junction usually found in interscapular BAT) were also described for the first time between brown adipocytes in browned periovarian WAT.

In a similar experiment in mice, Jimenez et al. (428) showed that the transformation of WAT into BAT-like tissue occur with an intermediate step in which adipocytes become multilocular and express UCP1 mRNA but not the protein. Importantly, the UCP1- multilocular cells appearing in WAT after cold stimulus are quite different in their size, morphology, and UCP1 protein expression from typical brown preadipocytes observed during BAT anlage development. On the other hand, these cells resulted similar to the intermediate forms between white and brown adipocytes found in the boundary areas between WAT and BAT and described in detail earlier (the adipose organ is mixed). Thus, while a convergence of data seemed to support the browning phenomenon and its variability in different depots and in mice with different backgrounds (364), the cellular basis of it remained unknown.

Data mainly from our lab supported the idea for a direct conversion of white to brown adipocytes (153, 158, 163, 164). The retroperitoneal fat of 20 weeks old rat is 100% composed by white adipocytes. We showed that administration of 1 mg/Kg of CL 316,243 (β3AR agonist) for 7 days induces a striking morphologic transformation of retroperitoneal fat in these old rats. About 17% of the parenchyma was transformed into a multilocular fat and about 8% of the multilocular cells present in this fat expressed UCP1. Bromodeoxyuridine (BrdU) is a substance that is incorporated into the DNA of replicating cells and that subsequently persists in the nuclei of the cells deriving from that replication and can be revealed by immunohistochemistry. BrdU experiments suggested that 95% of the multilocular adipocytes derive from direct conversion of white adipocytes (398). Furthermore, electron microscopy revealed a series of intermediate forms between white and brown adipocytes with a progressive increase in number and a progressive acquirement of typical shape of mitochondria in parallel with the approach to the typical brown cell morphology.

Importantly, some unilocular cell showed a clear thickened cytoplasmic rim with an impressive number of mitochondria exhibiting a morphology that can be considerate intermediate between that showed by white and that of brown mitochondria.

Five years later, Jean Granneman published BrdU data in substantial agreement with an 85% of the multilocular adipocytes possibly derived from a direct conversion of white adipocytes (354). In cold exposed mice, we confirmed the importance of β3 adrenoceptors (β3AR), showing the blunted phenomenon in β3AR-KO mice. Furthermore, we described all aspects (including electron microscopy and immunohistochemistry) of transitional forms between white and brown adipocytes. Paucilocular UCP1 immunoreactive adipocytes were described for the first time (36).

More recently, we performed a detailed quantitative analysis of the whole adipose organ in mice with two different

genetic background (614, 950). Our analyses performed in adult female mice (Sv129 and B6) maintained at 6°C for 10 days, showed that the browning phenomenon is more pronounced in B6 than in Sv129 in line with data obtained also in other's laboratories (364). Our quantitative work showed that the total number of brown adipocytes (defined as multilocular UCP1 immunoreactive adipocytes) increase about four times (compared with warm acclimated mice) in B6 mice (from 12% to 53% of the total number of adipocytes in the adipose organ). In Sv129 mice, the increase is also significant but limited to 0.3 times (from 45% to 60% of the total number of adipocytes in the adipose organ).

In both strains, the prevalent parenchymal cells of adipose organ after cold acclimation are brown adipocytes. White adipocytes represented in fact about only 35% (B6) or 20% (Sv129) of the parenchymal cells of the organ.

The rest of the parenchyma (10%-20%) of adipose organ, in these experimental condition, resulted composed by cells with intermediate phenotype between white and brown adipocytes: that is, multilocular UCP1- (adipocytes with brown-like morphology but lacking the brown molecular marker) and paucilocular UCP1+ adipocytes (adipocytes with white-like morphology expressing the brown molecular marker).

Of note, paucilocular UCP1+ adipocytes are much more frequently found in cold than in warm acclimated mice in line with the idea that browning is caused by a direct white to brown conversion.

Thus, after cold acclimation the main phenomenon in both strains was the increased number of brown adipocytes and the reduction in number of white adipocytes. Of note, in absence of a variation in the total number of adipocytes of adipose organ the increased number of brown adipocytes was equivalent to the reduction in number of white adipocytes. In theory, the disappearance of white adipocytes could be due to apoptotic phenomena, but, cold exposure is antiapoptotic (526,640) and detailed histologic analyses did not reveal any sign of apoptosis (401,455) or macrophages infiltration, suggesting a possible direct conversion of white to brown adipocytes in line with previous studies from our and other's laboratories.

Several data seem to converge on the idea that WAT browning is due to two main phenomena: direct progressive conversion of white or white-like adipocytes to brown adipocytes and neo-development of brown adipocytes (also called de-novo adipogenesis). These two phenomena are easily distinguished by morphology analyses. As a matter of fact, the direct conversion allows to observe (by electron microscopy and immunohistochemistry) the presence of the whole spectrum of intermediate forms between white and brown adipocytes, including the UCP1+ paucilocular cells (36, 166).

The neo-development allows to observe the whole spectrum of intermediate forms between poorly differentiated cells (progenitors or preadipocytes) and mature adipocytes. Although the precise cell type able to differentiate to a mature brown adipocyte is not univocally identified (see paragraph on origin of adipocytes), it is widely accepted that early stages of differentiation are easily recognizable by electron microscopy and well reproducible in primary culture since many years (37, 171, 622, 848).

Brown adipocytes at early stages of differentiation are poorly differentiated small cells (diameter around 5-10 μm) with all the morphologic and immunohistochemistry features described above (see paragraph on origin of adipocytes) (153, 169). These UCP1 immunoreactive small and poorly differentiated cells are well distinguishable from the UCP1 immunoreactive paucilocular cells that are close in size and morphology to mature white adipocytes (36).

Thus, morphometric ultrastructural analyses of WAT in cold exposed mice are able to distinguish between the two cellular components giving rise to the browning phenomenon and several data excluded any significant contribution of *de novo* adipogenesis, at least in the in the inguinal fat ((36, 202, 354, 398, 428, 950).

Furthermore, data *in vivo* (36,506) and *in vitro* (100,101) seem to suggest that the proliferation of brown adipocyte precursors is β1AR dependent, but only β3AR and not β1AR agonist administration is able to induce WAT browning in inguinal WAT.

In line with these data, we observed a significant increase of preadipocytes (*de novo* adipogenesis) after β1AR agonist administration without a WAT-browning and a significant increase of paucilocular UCP1+ adipocytes (direct WAT-to-BAT conversion) after β3AR agonist administration with WAT-browning in inguinal WAT of adult mice (36). Thus, β3ARs seems to play a pivotal role to mediate the browning through a direct white to brown conversion in line with data supporting that β3AR ablation blunt the browning phenomenon (36, 428, 882).

All these data seem to converge on the idea that browning of adipose organ of cold acclimated or treated with β3AR agonists rodents is mainly due to the plasticity of adipocytes.

The presence of β3ARs in mature white adipocytes could therefore serve as key player of their plasticity as shown by the almost absence of browning in WAT of cold exposed β3AR knockout mice. The appearance of paucilocular UCP1+ adipocytes with mixed mitochondrioma during WAT browning could represent a morphologic marker of white-brown conversion. These data are fully confirmed also by recent experiments of Granneman group (506, 507).

WAT browning is evident in both subcutaneous and visceral compartments, but epididymal, omentum, and retroperitoneum seems the visceral WAT more resistant to BAT conversion and we found delipidated cells without clear signs of brown conversion in these depots of rats and mice (153, 169).

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However, recent improvements of biotechnologies suggest that the appropriate technique able to demonstrate the direct white to brown conversion seems to be the lineage tracing experiments (854, 911).

Using this technique, Rosenwald et al. showed that cold-induced formation of UCP1+ multilocular adipocytes in WAT

of mice is reversed to adipocytes with the morphology and gene expression pattern of white adipocytes within 5 weeks of warm adaptation. Moreover, these white-typical adipocytes can convert into UCP1+ multilocular adipocytes on additional cold stimulation (763).

Wang et al. using a different mouse model with a similar lineage tracing technology outlined that cold induced WAT browning is also due to *de novo* adipogenesis, but essentially confirming an important component of direct conversion (959).

A further confirmation come from the recent work that elegantly demonstrated that PDGFRβ+ vascular mural cells (i.e., preadipocytes) do not significantly contribute to the initial cold induced browning of WAT, but only after prolonged cold exposure (949). However, a muscle-like origin for cells responsible of WAT-browning have also been proposed (539) and recently confirmed using a variety of Cre inducible mouse line (56).

Molecular mechanisms of WAT browning

White adipocytes are provided with β3ARs that increase in quantity after cold exposure (84, 224). The molecular signaling following activation of this receptor seems to inhibit a series of inhibitors of transcription factors important to drive the activation of thermogenic genes and therefore the brown phenotype (mainly PRDM16/PGC-1α and C/EBPβ with a series of complementary activators), thus the net result is a white to brown conversion of adipocyte (333). Thus, β3AR activated miR196a inhibits HOXC8/HDAC3 (Homeobox protein Hox-C8/Histone deacetilase 3) (112, 600) and the β3AR activated cAMP inhibits CK2 (casein kinase2) (832). β3AR signaling also inhibits MEF2C (myocyte-specific enhancer factor 2C) that activate miR133a/b (inhibitors of PRDM16) (914) (530). In addition, several other inhibitors are directly inactivated by the β3AR signaling such as 4EBP1 (924), SMAD3 (985), Rb/p107 (272, 381, 383), RIP140 (149, 463), and H3K27me3 (672).

A recent work supports also a role for mTORC1 in the β3AR signaling for browning (529). Furthermore, the LC3-dependent autophagy mechanism, that seems to be important for maintenance of the white phenotype and that is under the control of mineralocorticoid (MR) receptor activity (15), can be inhibited by the β3AR signaling trough the TASK1 potassium channel (Twik-related acid-sensitive K+ channel) activity (692).

Together with the inhibition of inhibitors of brown phenotype β3AR signaling induce the synthesis of FGF21 (Fibroblast Growth Factor 21) (291, 947) that reinforce the browning phenomenon by an autocrine mechanism trough FGF21R/β-Kloto receptor (342). A recent paper supports a role for FGF-21 also in the activation of immune-mediated WAT-browning (see the succeeding text: immunebrowning paragraph) (412). Furthermore, β3AR signaling activates also signals that directly act on positive browning factors PGC-1α such as COX2 (555, 942). In line with these data, the presence of inhibitors that, if removed, allow conversion of myoblast to brown adipocytes has been recently proposed (954).

Thus, the white adipocyte could be considered as a masked brown adipocyte that reveal its true face after noradrenergic activation of its β3ARs and removal of molecular inhibitors (Fig. 12), but a recent paper seems to support a dispensable role for β3ARs in browning of FVB/N mice (221).

Which other data support the idea that a white adipocyte is a masked brown adipocyte?

Some authors believe that a subpopulation of "white-like" adipocytes are in some way more prone to convert directly to "brown-like" adipocytes (often called brite or beige, see the succeeding text), but morphologic and immunohistochemistry *in vivo* experiments comparing the inguinal WAT after fasting, refeeding, and cold exposure showed that most unilocular adipocytes (i.e., with the anatomy of white adipocytes) in this depot, able to undergo the slimming process (i.e., changing their morphology into a typical delipidated cell type when the animal is fasted) and able to refill with lipids their cytoplasm if the animal is refeeded (thus, showing the classic white adipocyte physiology properties) are also able to convert to thermogenic multilocular UCP1 immunoreactive (thus, true brown) adipocytes (36, 168, 606).

Some visceral areas of murine adipose organ are less prone to browning phenomenon, but this assumption largely derive from the data obtained with epididymal visceral fat (easy to sample and therefore widely used), but many visceral depots (also in humans) are composed by BAT or convertible WAT (all areas of adipose organ surrounding aorta and its main branches: subclavian, carotid, intercostal, renal, and mesenteric) (212, 950). Thus, the anatomical site *per se* do not define the proneness of WAT to BAT conversion and the *in vitro* experiments claiming the cell autonomous properties of adipocytes should consider the level of genetic "influence" made on that specific depot by the parenchymal nerve fibers activity in that specific experimental model.

Browning and noradrenergic parenchymal nerve fibers plasticity

A strong positive correlation between browning and density of noradrenergic parenchymal nerve fibers (NE fibers) has been described (950) (Table 3).

ASC: In Sv 129 mice, the density of parenchymal tyroxine-hydroxilase (TH) immunoreactive nerve fibers (NE-D) increase four times in this depot (mainly composed by: interscapular, subscapular, cervical, and axillar-thoracic fat) in cold acclimated mice (C-mice) versus warm acclimated mice (W-mice) (from ≈12 to ≈45 fibers/100 adipocytes). Of note, this depot is composed mainly by brown adipocytes (UCP1+ multilocular cells, ≈80%) in W-mice and cold acclimation increased only the number of multilocular UCP1-adipocytes. Of course, brown adipocytes were more intensely UCP1 immunostained and presented smaller lipid vacuoles in all areas studied of cold acclimated mice.

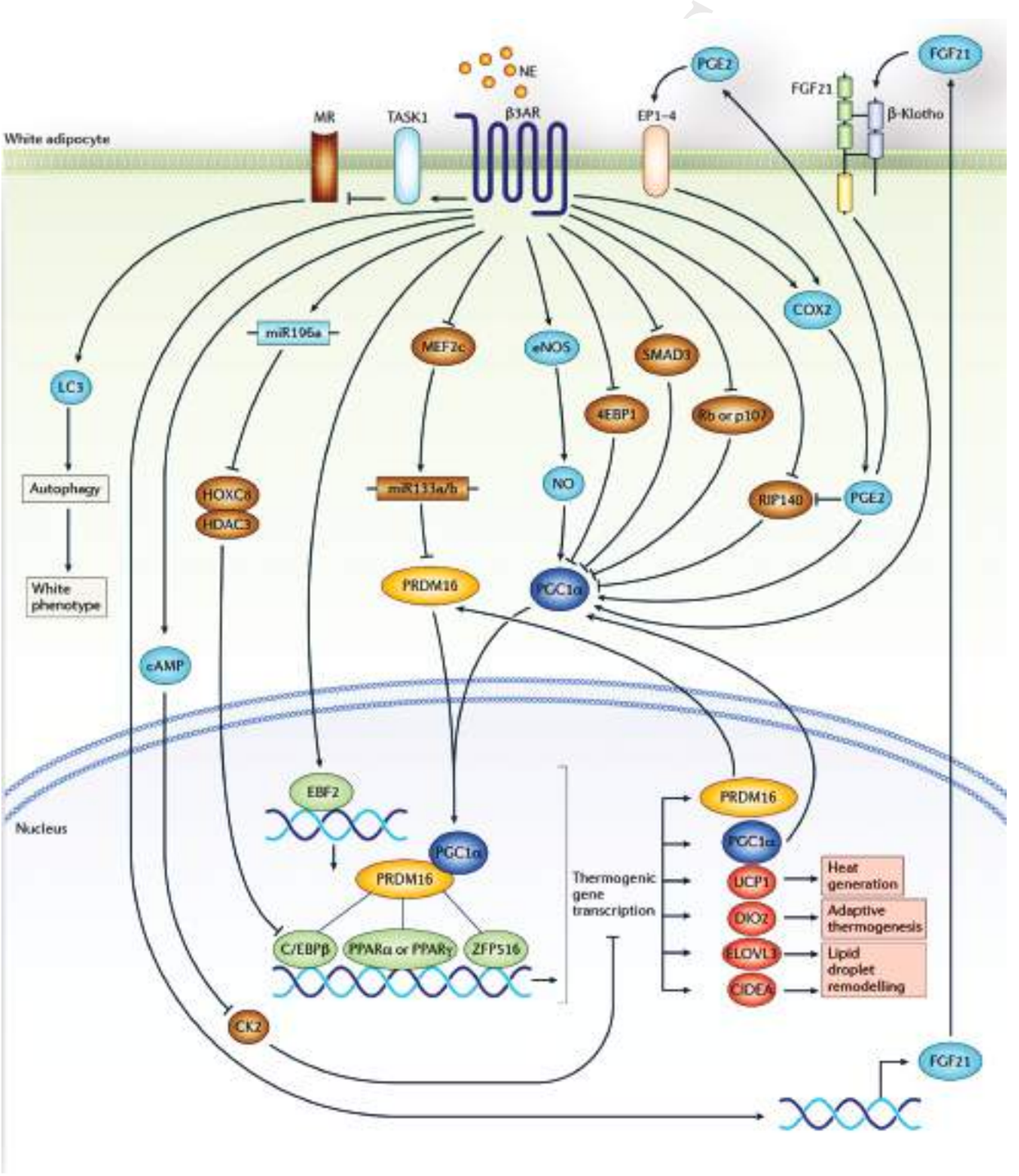

Figure 12 Potential β_3-adrenoceptor-dependent molecular mechanisms driving white-to-brown adipocyte transdifferentiation. All brown-colored molecules are inhibitors of brown phenotype and are inhibited by activated β_3-adrenoceptor signaling. Adapted, with permission, from (333).

Table 3 Adipose Organ Composition and Parenchymal Nerve Fibers Density in C57BL/6J and Sv129 Mice; Adapted, with Permission, from (948)

	Anterior subcutaneous	Posterior subcutaneous	Mediastinal	Mesenteric	Retroperitoneal	Abdominopelvic
C57BL/6J						
No. unilocular adipocytes						
28°C	$56 \pm 8^{\#\#\#}$	14 ± 1	$6 \pm 3^{\#\#}$	12 ± 1	2 ± 1	27 ± 2
6°C	$25 \pm 5^{*\#\#}$	16 ± 3	0.6 ± 0.3	$10 \pm 1^{\#}$	2 ± 0.2	$15 \pm 4^{*}$
No. UCP1-negative multilocular adipocytes						
28°C	$8 \pm 0.3^{\#}$	1 ± 0.2	$0.2 \pm 0.08^{\#}$	0.7 ± 0.4	0	$5 \pm 0.8^{\#}$
6°C	$6 \pm 1^{\#\#}$	$2 \pm 0.4^{*}$	$3 \pm 0.1^{\#}$	$1 \pm 0.3^{\#}$	0.4 ± 0.1	11 ± 0.8
No. UCP1-positive multilocular adipocytes						
28°C	$12 \pm 4^{\#\#\#}$	0	8 ± 3	0	0	0.7 ± 0.4
6°C	$54 \pm 4^{**\#\#\#}$	$0.6 \pm 0.2^{\#\#}$	7 ± 1	0.003 ± 0.002	0.001 ± 0.0002	$7 \pm 2^{*\#}$
TH–positive fiber density						
28°C	11 ± 2	2 ± 0.8	$34 \pm 0.7^{\#\#\#}$	$6 \pm 2^{\#\#\#}$	12 ± 1	16 ± 1
6°C	$61 \pm 5^{**\#}$	$6 \pm 2^{\#\#}$	50 ± 9	$9 \pm 2^{\#\#\#}$	7 ± 2	$31 \pm 3^{*}$
SV129						
No. unilocular adipocytes						
28°C	16 ± 3	19 ± 0.2	$0.1 \pm 0.1^{\#}$	10 ± 0.0	2 ± 0.5	22 ± 1
6°C	$9 \pm 2^{*}$	$16 \pm 1^{*}$	0	$4 \pm 2^{**}$	2 ± 0.6	$9 \pm 1^{***}$
No. UCP1-negative multilocular adipocytes						
28°C	2 ± 0.3	0.9 ± 0.5	0.05 ± 0.03	0	0.1 ± 0.1	9 ± 2
6°C	$11 \pm 3^{*}$	$4 \pm 1^{*}$	0.1 ± 0.04	$10 \pm 2^{**}$	0.1 ± 0	$7 \pm 1^{\#}$
No. UCP1-positive multilocular adipocytes						
28°C	73 ± 6	0	5 ± 1	0	0.006 ± 0.006	15 ± 3
6°C	73 ± 5	$6 \pm 1^{***}$	9 ± 2	$0.2 \pm 0.03^{*}$	$0.4 \pm 0.1^{**}$	$25 \pm 3^{*}$
TH–positive fiber density						
28°C	12 ± 7	1 ± 0.2	78 ± 2	1 ± 0.1	0.2 ± 0.1	16 ± 2
6°C	$45 \pm 8^{*}$	$12 \pm 2^{**}$	72 ± 26	$20 \pm 2^{**}$	2 ± 0.9	$38 \pm 0.8^{**}$

Data of obesity-resistant Sv129 mice from a previous work (26) are provided for comparison. Number of adipocytes × 10^6 TH-positive fiber/100 adipocytes.
Indicates statistically significant difference between temperature conditions (same depot, same strain). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
$^{\#}$Indicates statistically significant difference between strains (same depot, same temperature condition). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.
Total number of adipocytes contained in the adipose organ of C57BL/6J and Sv129 results unchanged. $P = 0.25$ in 28°C mice; $P = 0.18$ in 6°C mice.

In B6 mice, NE-D in ASC increased about six times (from ≈11 to ≈60 fibers/100 adipocytes). Thus, the NE-D in Sv129 and B6 W-mice was quite similar but in B6 mice, this depot was composed mainly by white adipocytes (≈75%). Cold adaptation induced an increase of brown adipocytes number of about five times (B6), thus the ASC of two strains have striking differences both in unstimulated and stimulated situations, but in both cold induced a relevant increase of parenchymal noradrenergic nerve fibers density accompanied by a remodeling of the tissue with increased signs of BAT activity and increased number of multilocular adipocytes (Sv129) and brown adipocytes (B6).

PSC (composed by dorso-lumbar, inguinal, and gluteal parts) is mainly formed by white fat in both strains in W-mice the NE-D is sparse (1-2 fibers/100 adipocytes). Cold induced 12 times increase of NE-D in Sv129 and 3 times increase in B6 mice accompanied again by WAT remodeling with increase of brown adipocytes (from 0 to 6×10^6) in Sv129 and of multilocular UCP1- adipocytes (from 0 to 0.6×10^6) in B6 mice.

Mediastinal depot is highly innervated. In W-mice, the NE-D reach the maximal levels (about 75/100 and 35/100 adipocytes in Sv129 and B6, respectively). In line with these high NE-D, the % of brown adipocytes is the highest in both

strains (near 100% and 75%, respectively). In C-mice, the NE-D did not change in Sv129 (72-78 fibers/100 adipocytes) and increased in B6 (from 34 to 50 fibers/100 adipocytes) and an increase of only multilocular UCP1- (from 0.2 to 3×10^6) was observed in B6 mice. In Sv129 mice, the number of brown adipocytes increased from 5 to 9×10^6 in absence of an increase of NE-D.

Mesenteric depot showed low NE-D in both strains (1 fiber/100 and 6 fibers/100 adipocytes in warm Sv129 and B6 respectively). In postcold mice, the increase of NE-D was 0.3 times in B6 with a slight increase in brown adipocytes (from 0 to 0.003×10^6), but in Sv129 mice, the increase of NE-D was near twenty times. In these last mice, the remodeling of WAT was also striking and the % of unilocular white adipocytes that were near 100% in W-mice reduced to about 10%, the rest was formed by multilocular UCP1- adipocytes.

Retroperitoneal depot is poorly innervated in both strains, NE-D increased after cold but remodeling was very modest.

Abdomino-pelvic depot (composed by perirenal, periovarian, parametrial, and perivesical fat) is quite well innervated mainly in the perirenal area. NE-D is 16 fibers/100 adipocytes in both strains and increased in both about two times in C-mice with an important remodeling of adipocyte type composition: from about 50% of multilocular adipocytes (50% UCP1+) to about 80% (80% UCP1+) in Sv129 and from about 20% of multilocular adipocytes (10% UCP1+) to about 50% (40% UCP1+).

In synthesis in both strains cold acclimation induced increased density of parenchymal NE fibers in almost all fat depots studied (in the whole organ the increase was 1.75 times in Sv129 and 2.3 times in B6) with an almost regular parallel increase in number of multilocular and brown adipocytes. Moreover, unilocular white adipocytes reduced their number of an amount equivalent to the increase of multilocular and brown adipocytes. Of note, remodeling of fat included also an increased density of capillaries network that was not quantified.

Thus, the different browning propensity of different fat depots in the two strains seems to be mainly linked to their noradrenergic parenchymal innervation and to the strain specific ability to increase the density of noradrenergic parenchymal fibers. The most reactive subcutaneous depot was the PSC in Sv129 and the ASC in B6 mice.

All visceral fat depots were highly reactive in both strains, but the mesenteric retroperitoneal and perivesical resulted more plastic in Sv129 (10, 11, 212, 364, 476, 611, 950).

Immunebrowning

Data from our and other's laboratory seems to converge on the idea that plasticity of peripheral nerve fibers of SNS is of pivotal importance for the WAT browning phenomenon. As a matter of fact, the density of noradrenergic parenchymal fibers (TH immunoreactive) in WAT increase in parallel with browning and a positive correlation between number of brown adipocytes and density of noradrenergic parenchymal nerve fibers have been found (see previous paragraph) (611). Thus, even the less innervated part of the adipose organ, that is, the most "white" parts mainly (but not exclusively) composed by white adipocytes, recruit parenchymal noradrenergic nerve fibers during WAT browning and the newly browned areas are always densely innervated. Furthermore, the importance of parenchymal nerve fibers is outlined by the observation that surgical denervation blunts the browning phenomenon even in the most reactive part of the organ such as the inguinal depot of Sv129 mice (a very reactive strain) (506).

Nevertheless, other mechanisms seem to parallel the nerve plasticity and interstitial cells owning to the innate immune cell system seems to play a major role (960).

Cold exposure induces recruitment of eosinophils and of M2 macrophages in subcutaneous WAT (972).

Eosinophils are granulocytes with an important functional role in allergy and in parasitism state; they are sparse in blood of people in western developed countries but abundant in people in less developed countries where parasitism is frequent and metabolic syndrome rare (448). In WAT of lean mice, they are quite abundant and represent about 4% to 5% of SVF cells (972). Eosinophils are the major source of IL-4 that is responsible for activation of M2 macrophages. It has been claimed that IL-4 induces increase of TH (the rate limiting enzyme necessary for all catecholamine synthesis) in M2 macrophages and this is accompanied with a WAT browning dependent from all these components of the T2 immune cell system (632, 712). Of note, this immune-system mediated mechanism of WAT browning is absent in interscapular BAT and seems to be restricted mainly to subcutaneous fat. The elegant studies demonstrating the immune-mediated browning of subcutaneous fat showed that this phenomenon is metabolically relevant inducing increase of energy expenditure and ameliorating metabolic dysfunctions in models of obesity (503).

IL-33 is a cytokine able to activate ILC2s that are lymphocytes able to regulate several types of immune responses. ILC2s are present in WAT and their secretion of IL-5 and IL-13 sustaining eosinophils and M2 macrophages promote glucose homeostasis (379, 589, 595, 648). It has been shown that administration of IL-33 results in subcutaneous WAT browning trough recruitment and activation of the cytokine cascade network involving the ILC2s/eosinophils/M2 system (96). Activated ILC2s produce IL-13 and activate eosinophils to produce IL-4. Both cytokines are able to directly recruit PDGFRα+ adipocyte precursors (expressing the IL-4Rα necessary to respond to both IL-4 and IL-13) developing into adipocytes with brown phenotype, thus contributing to the WAT browning phenomenon (503). Interestingly, this cytokine-mediated mechanism of PDGFRα+ adipocyte precursors recruitment plays a role also during physiologic postnatal development of brown committed adipocytes in subcutaneous fat (503).

The physiologic source of IL-33 contributing to the browning phenomenon is not well established, but it has been

shown that human white adipocytes and preadipocytes *in vitro* and *in vivo* are able to produce this cytokine and are provided with its receptors (970).

Furthermore, ILC2s have been identified also in human WAT and a decreased activity has been shown in WAT of obese mice and humans and it has been shown also that endogenous IL-33 is necessary to limit spontaneous obesity and white adipocyte hypertrophy (96) in line with data showing a protective role of IL-33 in obesity and related disorders (591, 662). These Authors also demonstrated that ILC2s are also able to sustain a WAT browning independently from the activation of eosinophils and M2 macrophages. This direct WAT browning activity seems to be due the opioid-like methionine-enkephalin (MetEnk) peptides production by ILC2s lymphocytes acting on specific opioid receptor δ 1 present in subcutaneous white adipocytes (96).

Surprisingly, recent data obtained in mice with different genetic background in six different independent laboratories seems to deny any role for M2 macrophages in catecholamine secretion and WAT browning. Using mice lacking TH in peripheral tissues (THΔPER), Fischer et al. (2017) showed that irradiated mice with reconstituted myeloid population from THΔPER or WT mice had identical thermogenic metabolism. Furthermore, immunohistochemistry and HPLC experiments failed to reveal any significant amount of TH and catecholamine in macrophages (290).

These conflicting results are difficult to understand (726) and for sure need further studies, but a very recent result seems to support a functional link between macrophages and nerves in BAT activity, thus a role for macrophages and other cell type of innate immunity in the WAT browning phenomenon cannot be definitively excluded so far (969).

Purinergic browning

Browning mechanisms alternative to the classic noradrenergic/β3AR pathway are strongly hoped for future therapies of obesity and related disorders in view to the fact that β3AR agonists produced in the past are effective for small mammals but not for humans (496, 935). The last generation β3AR agonist mirabegron is able to activate human BAT (212) but it has been approved for clinical use only for overactive bladder allowing to suspect that clinical trials for obesity failed.

In this contest, it is interesting to note that mice lacking all beta-adrenergic receptors (β-less mice) are able to reconstitute their BAT (transformed into a WAT-like in these mice) (26) under a chronic subordinate stress stimulus (723). In this experimental condition, sympathetic parenchymal nerve fibers density increase without a testable contribution of adrenergic signaling to browning. The immunoreactivity of nerve fibers for VNUT (vesicular nucleotide transporter, which is required for ATP storage in secretory vesicles) (391), together with its upregulation in stressed mice, suggest a purinergic signaling in these experimental conditions. Of note, VNUT immunoreactive nerve are present also in human BAT (723). These data are in line with the observation that adenosine activates human and murine brown adipocytes at low nanomolar concentrations acting on A2A receptors and that A2A agonists induce browning of white adipocytes. Importantly, the WAT browning induced by A2A agonists reduces fat mass and improve glucose tolerance in diet-induced obese mice (345).

Beige, brite, or brown?

This newly formed BAT-like tissue has been also denominated as beige or brite (brown-like in white) or inducible brown fat (196, 686, 973). These denominations have been proposed mainly to underline the unusual presence of brown adipocytes in predominantly white fat depots and to emphasize the molecular differences between the "classic" interscapular BAT and the WAT transformed into a BAT-like tissue by, mainly, adrenergic activators.

The widespread idea that interscapular BAT is indeed the anatomical site of "classic BAT" derive from its anatomical structure (a compact tissue composed almost exclusively by UCP1 immunoreactive brown adipocytes) and several other aspects:

1. This is the site of the first anatomical descriptions (920),
2. The vast majority of BAT studies are performed in this tissue,
3. It is present in most of the small mammals used in experimental protocols (700),
4. It persists in older animals (605, 793),
5. It expresses quite specific ontogenetic markers: En1 (engrailed 1), Myf5 (myogenic factor 5), and PAX7 (paired box7) (808, 835), and
6. It expresses a marker gene (Zic1) that is not expressed by browned WAT (220).

In my opinion, none of the aforementioned reasons allows distinguishing brown adipocytes found in interscapular area from those found in other regions in the adipose organ. Historical anatomic and physiologic studies teach us that a cell and its appropriate denomination should consider mainly its anatomy and function. Until now, no data contrast with the fact that multilocular UCP1+ adipocytes have thermogenic properties wherever their anatomical location would be in the adipose organ. There is no doubt that adipocytes with intermediate morphology between white and brown exist and that their number increases during browning (see detailed description in previous paragraphs). Thus, it is not surprising that gene expression analyses comparing interscapular BAT (where brown adipocytes form a quite compact tissue that is almost uniform in its morphology), with browned white

fat (where brown adipocytes are only a percentage of the mixed tissue), show quite relevant gene expression differences. Furthermore, together with white and brown adipocytes these browned white fats usually contain also all the range of adipocytes with intermediate morphology between white and brown phenotype offering an explanation to intermediate and variable gene expressions. In this context, it is interesting and in line with the above considerations, that during brown differentiation *in vitro* of PDGRα+ adipocyte precursors from inguinal fat the so called "beige/brite" gene expression markers (Tnfsrf9, Klhl13, and Tmem26) are downregulated (503), suggesting that these markers work better for the identification of adipocytes that are not jet fully differentiated, that is, for example: adipocytes in the intermediate steps of browning differentiation.

In B6 mice, the browning phenomenon is quite consistent in ASC where cold acclimation induces five times increase in the number of brown adipocytes (compared to warm acclimated). Thus, the newly formed brown adipocytes own to the same anatomically well-defined depot of adipose organ including the "classic" interscapular BAT and its white periphery is a site of a striking WAT browning. The newly formed brown adipocytes in this area that surround the more central part of the interscapular BAT seems quite identical to classic brown adipocytes including their positional marker Myf5 lineage (783).

In conclusion, a specific cell type that can be described as beige/brite, to date is not precisely defined and I think that considering all together the most reasonable conclusion should be to restrict the definition of beige/brite adipocytes to those with a paucilocular morphology, that is, those that are in the intermediate step of differentiation between white and brown adipocytes. Moreover, this cell type when stained by UCP1 antibodies in immunohistochemistry stain beige (851) (Fig. 13).

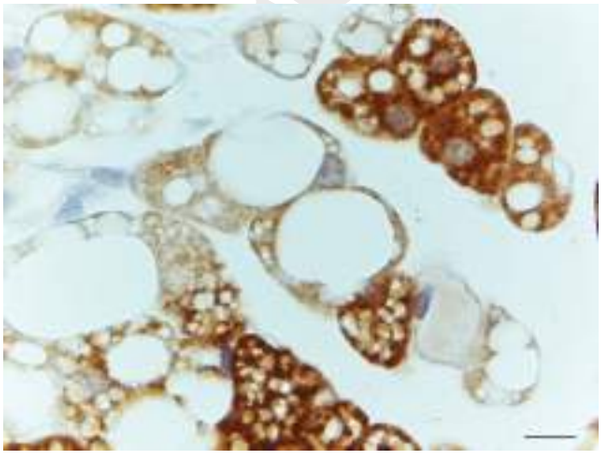

Figure 13 White adipocytes converting to brown adipocytes show a paucilocular morphology and a weak UCP1 immunoreactivity (Beige adipocytes). Bar: 9.0 μm. Adapted, with permission, from (851).

Furthermore, the concept of adipose organ as detailed in this and in previous reviews imply the mixture of WAT and BAT as a basic conceptual aspect of the anatomy and physiology of this organ: the mutual reversible conversion of the two tissues as a reservoir of energy partitioning toward thermogenesis (browning) during chronic cold exposure or toward storing (whitening) in case of chronic positive energy balance.

The healthy properties of BAT and browning

Whatever should be the correct denomination of newly formed multilocular UCP1+ thermogenic adipocytes data are widely converging on their healthy properties both in small mammals and in humans (627). Several experimental data showed that removal of BAT or its βARs result in obesity and its related disorders (26, 186, 545, 809). On the other hand, BAT activation and browning of adipose organ are highly effective to combat obesity and its related disorders including T2 diabetes and atherosclerosis: that is, the metabolic syndrome (625). Thus, it is not surprising that BAT activation and browning are also efficient in prolonging the lifespan (659, 660).

In this view, several studies have been made to find molecular targets for therapeutic strategies alternative to the physiologic adrenergic signaling that could interact with the cardiovascular system.

Among those described in the molecular mechanisms of WAT browning (see the preceding text), recent data seem to support a role for intestinal microbiota.

Microbota and WAT browning

Since the seminal work of Nicholson et al. (634), it became evident a relationship between microbiota and host metabolic interactions and in 2006 Turnbaugh et al. and Ley et al. (516, 926) showed that intestinal microbiota of obese animals has peculiar characteristics and germ free animals transfected with this characteristic microbiota increase their fat mass suggesting a link between microbiota and adipose organ physiology.

Chevalier et al. (142) recently found a relationship between thermogenesis and intestinal microbiota composition. Cold exposed mice showed a major shift in proportions, especially in the ratio Firmicutes/Bacteroidetes where Firmicutes abundance increased over Bacteroidetes with a reduction of Verrucomicrobia phylum. Transfecting this microbiota from cold exposed to germ-free mice induces fat loss and improvement of glucose and insulin metabolism. Subcutaneous and visceral fat of these animals show smaller adipocytes and WAT browning.

Furthermore, Suárez-Zamorano et al. (874) showed similar results of WAT browning and amelioration of obesity by alteration of microbiota trough antibiotics administration.

The mechanisms for this microbiota-induced WAT browning are unknown but several hypotheses have been proposed. Gut microbiota are able to modify the bile acids pool

(738, 739) and brown adipocytes bear the bile acids receptor TGR5 that can be activated also in humans with increase of energy expenditure (99). Furthermore, activated intestinal TGR5 mediates synthesis and secretion of the intestinal incretin GLP-1 (glucagon-like peptide 1) that, together with its glucose-dependent insulinotropic properties, can be responsible for a central stimulus of thermogenesis (48, 898). Finally, the short chain fatty acids (SCFAs) are the main product of gut microbiota fermentation and SCFAs can directly activate BAT and WAT browning or exert indirect influence through a stimulus for GLP-1 production by intestinal L cells (449, 774, 901).

Physical exercise and WAT browning

Physical exercise induces size reduction and mitochondrial biogenesis in adipocytes of WAT (203, 863). This well-known and relatively old observation can now be viewed as an early step of WAT browning and several healthy consequences of physical exercise (82) can be attributed to this phenomenon. The mechanisms involved include the activity of several actors. Central nervous system acts with a direct increase of activity and with expansion of its sympathetic branch.

SNS

Mimicking cold exposure physical exercise induces increase of parenchymal nerve fibers density in the adipose organ (227). Interestingly, the same amount of physical exercise induces more browning in animals maintained in an enriched environment. In these animals, the increased BDNF hypothalamic production seems to be responsible for the enhanced browning (113).

Several cytokines or myokines produced by active skeletal muscles have been suggested to play a role in WAT browning (782, 864).

IRISIN

Bostrom et al. showed that transgenic muscles engineered to mimic trained muscles upregulated the gene expression of fibronectin type III domain containing 5 (FNDC5), which after cleavage is secreted into the blood stream as irisin (86, 504). Data suggest that irisin is also produced by both subcutaneous and visceral WAT (743). The browning role of irisin is controversial (8, 262, 263, 646, 722) and recent data suggest that another physiologic target for this hormone could be the locomotor apparatus itself (188-191).

Natriuretic peptides

Natriuretic peptides are hormones produced by heart with the main purpose to maintain homeostasis with regard to blood volume, blood pressure, and salt balance (492, 604, 786). The adipose organ influences the activity of these peptides by clearance receptors. On the other hand, natriuretic peptides acting on functional receptors increase cyclic GMP levels to activate cGMP-dependent protein kinase and activation of p38MAPK, thus their activity could be synergic with that of classic β-adrenergic receptor stimulation (192). Bordicchia et al. showed that natriuretic peptides promote browning of human white adipocytes and browning in treated mice (83, 193).

IL-6

IL-6 is produced by skeletal muscles and other organs (including fat). Its production increase during exercise (678-680) and in its overexpression in mice increase BAT activity (549). Recently a WAT browning effect of IL-6 has also been proposed (1).

BAIBA

Roberts et al., using a metabolomics approach, showed that exercised skeletal muscles secrete the myokine β-aminoisobutyric acid (BAIBA) (742). BAIBA induces WAT browning and β-oxidation in hepatocytes both *in vitro* and *in vivo*. Furthermore, this myokine induces a brown adipose-like phenotype in human pluripotent stem cells, and improves glucose homeostasis in mice. Interestingly, in a large human cohort study (Community-based Framingham Heart Study), plasma BAIBA concentrations resulted increased with physical exercise and inversely associated with metabolic risk factors (742).

Metrnl

Meteorin-like (Metrnl) is a circulating factor that is induced in muscle after exercise and in WAT after cold exposure (720). Increasing serum levels of Metrnl stimulates WAT browning. Metrnl stimulates an eosinophil-dependent increase in IL-4 expression and promotes alternative activation of adipose tissue macrophages, which are required for the increased expression of the thermogenic gene programs in fat (see also immunebrowning paragraph).

Finally, explanted subcutaneous fat of trained mice into sedentary animals improved their glucose tolerance and glucose uptake in muscle suggesting the possibility that trained subcutaneous fat could release adipokines that could reinforce the healthy WAT browning phenomenon (864).

Other Nonthermogenic Functions

Physical exercise induces WAT browning, but some data support an activation of BAT without an increase of its thermogenic gene expression (227). These data suggest that BAT could exert other functions and the increased expression and membrane localization of MCT-1 (proton-linked monocarboxylate transporter) strongly suggest a role of BAT in trained animals in the lactate metabolism (227). Interestingly lactate induces FGF21 and both play an important role in

WAT browning (427). It has been suggested that browning induced by lactate and other catabolites such as the ketone body β-hydroxybutyrate could represent an adaptive mechanism to alleviate redox pressure (122, 862). Adiponectin (see endocrine paragraph) is a circulating hormone with antiatherosclerotic properties that is produced by both WAT and BAT, and the BAT production is not linked to its sympathetic activation (707).

A direct influence on cardiovascular system is also exerted by adipose organ expression of clearance receptor of atrial natriuretic peptide (NPr-C). Fasting induces a dramatic suppression of NPr-C gene expression in both WAT and BAT that appears to be accompanied by an increased biological activity of ANP (785). Thus, the natriuresis and diuresis and reduction of blood pressure induced by fasting might result from a reduced expression of NPr-C in adipose organ.

Some data also suggest that browning support an anabolic influence on bone system probably through the production of insulin-like growth factor binding protein 2 (IGFBP2) (714).

Metabolism

The WAT specific main function of adipose organ is to allow survival in the intervals between meals (760, 860).

For millions of years, until about 100 years ago in western countries, it was necessary to spend a lot of time and energy to find food for survival and the presence of a large energy storage depot in WAT of adipose organ was essential to guarantee survivals of humans and other mammals. The metabolic needs of the cells can count on an energy reserve in the WAT component of this organ able to guarantee up to several weeks' survival without any need for new fuel intake. Old experimental evidence shows lean persons surviving to up to 45 days of absolute fasting (454). But adipose organ also provides a strong endocrine stimulus to induce the search for additional source of energy: leptin (1013) (see also next paragraph). White adipocytes have the ideal shape for their physiology: spherical (maximal volume in minimal space) and are able to transform their morphology from spherical cells into elongated small fibroblast-like cells (slimmed cells) when the organism requires fuel (such as during prolonged fasting periods). The morphologic physiologic changes of adipocytes are accompanied by different phases of synthesis and secretion of leptin that is produced in positive relationship to the adipocyte cell size (540, 856). This hormone hematic concentration is proportional to the WAT in the adipose organ and when the energy stored in the organism tend to be dangerously low, then the leptin low level in blood assumes the role of strong stimulus for food search and intake by activation of various sites of limbic system in central nervous system provided with specific leptin receptors (201, 225) Thus, the physiologic fasting period between two meals have two main consequences: the use of stored lipids for metabolic needs and the stimulus for the brain to guarantee a behavior for food search and intake and consequent survival. The stimulus strength will depend on the fasting period and amount of WAT (249).

On the other hand, the organ cannot refuse the request of fuel storage such as during chronic positive energy balance periods and whitening of the organ (BAT to WAT conversion) together with white adipocyte hypertrophy and hyperplasia is one of the mechanisms that help in building up new parenchymal cells with lipid-storage capacities (26, 177, 280).

Thus, the reciprocal physiologic and reversible ability of conversion between WAT and BAT is an important intrinsic physiologic property of this organ (163, 164). Of note, the lipolysis due to fasting seems to be orchestrated by a sympathetic nervous system activity similar to that operating during cold exposure, but the cellular effects on WAT diverge strikingly (334). As a matter of fact, cold activated white adipocytes convert into brown adipocytes, but fasting activated white adipocytes transform into slimmed cells (158, 166). The relevant difference in the hormonal environment in the two conditions could play a role in the different biological effect on adipocytes. In particular insulin and natriuretic peptides could be important actors because insulin low levels in fasting could account for a major lipolytic effect of noradrenaline (17, 488, 491) and the inhibition of natriuretic clearance receptor expression without any reduction of the functional receptor in the adipose organ in fasting conditions could account for a reinforcement of the noradrenaline-induced lipolysis with consequent slimming effect on white adipocytes (83, 192, 239).

On the other hand, the noradrenergic pathway inducing BAT thermogenesis and browning is not only due to noradrenaline. It has been recently shown that cold is more efficient in BAT activation (as revealed by PET analysis) than direct noradrenaline administration to humans (209). Furthermore, beta less animals (i.e., mice lacking all types of βARs) can be induced to thermogenesis and browning (723) (see also purinergic browning paragraph).

Endocrine properties

The adipose organ produces and secrete a large number of factors with hormonal, autocrine and paracrine properties, and cumulatively called adipokines (Fig. 14).

Proteomic studies showed that adipose organ produces about 600 different adipokines (512). Most of them are produced by white adipocytes with differences between subcutaneous or visceral depots, but some are produced by brown adipocytes (138, 278, 343, 440, 456, 918). Here a short description of the most studied adipokines is reported because several are directly involved in adipose organ remodeling or remodeling *per se* can influence their production and functional role.

Leptin It is a 16-KDa protein with structural homology to cytokines, produced mainly by subcutaneous white adipocytes (301, 302, 1013). Its production is in relationship with size and number of white adipocytes in the organ (300, 610). It is produced with a circadian rhythm with apex in the late evening-midnight, in relationship to food intake

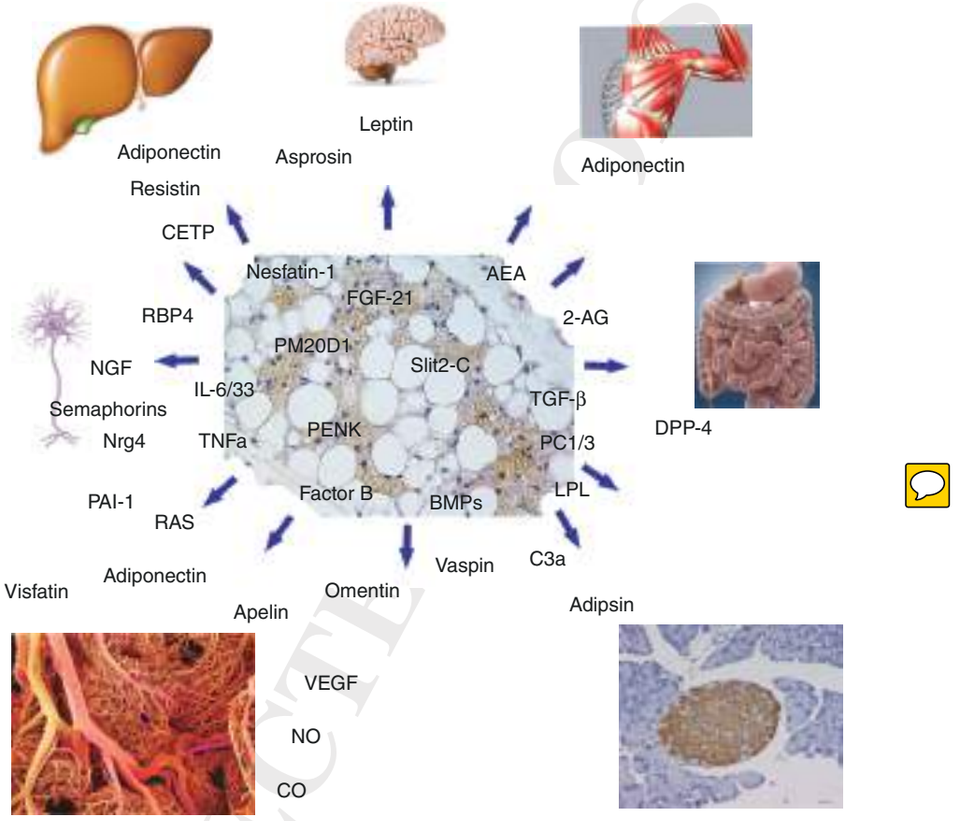

Figure 14 Graphical summary of the most important hormone-like molecules secreted by adipose organ.

timing. The intracellular localization of leptin has never been established definitively (315).

Leptin secretion is also dependent from several factors: such as insulin, glucocorticoids, TNFα, estrogens, C/EBPα that are positive inducers and β3AR activity, androgen, free fatty acids, and PPARγ agonists that are negative stimuli (564, 675).

Its principal functional receptor (Ob-RL member of the superfamily of cytokine receptors) is found mainly in the brain (hypothalamus and limbic system) where leptin exert its positive action on anorexigenic neurons and sympathetic neurons, but the most important behavioral effect is due to its low plasma level that induce a strong stimulus for food search and decrease in energy expenditure (249, 892). Mice and humans lacking leptin or its receptor are massively obese and their behavior denotes an irresistible need for food search and intake (184, 596, 649, 695). Most of the obese persons are leptin resistant (557), but rare genetic obesity due to lack of leptin production can be restored by recombinant leptin administration (277).

Leptin also regulates several neuroendocrine activities mainly acting on CRH, TRH, and GnRH hypothalamic neurons (394, 456, 494).

Leptin replacement during fasting prevents starvation-induced changes in the hypothalamic-pituitary-gonadal and pituitary-thyroid axes in healthy men (131). Several peripheral tissues express the leptin Ob-RL including: WAT, gonads, lung, placenta, adrenal medulla, liver, pancreas beta cells, jejunum, skeletal muscles, heart, cartilage, and blood mononuclear cells (226, 951). It has been calculated that the total amount of peripheral Ob-R account for less than 10% of the total and the physiologic role of these receptors remain to be elucidated although the old notion that female fertility is linked to a minimum amount of stored WAT (303) could be in relationships with the gonadal expression of Ob-RL and leptin replacement therapy can restore gonadotropin pulsatility in women with hypothalamic amenorrhea (965), but Kawwass et al. recently critically reviewed this topic (450).

Several other endocrine effects include regulation of immune function (mainly associated with malnutrition), hematopoiesis (proliferation and differentiation of hematopoietic cells), angiogenesis (91, 542, 564, 675). The effects of leptin on bones is quite controversial. Leptin-deficient ob/ob mice have increased bone mass, despite hypercortisolemia and hypogonadism (185, 253, 689, 927).

Data suggest that Leptin-responsive neurons in the VMH the ventral medial hypothalamus (VMH) are involved in leptin's effect on bone mass (885). Indeed, mice with defective SNS activity have high bone mass and are resistant to the antiosteogenic effects of leptin, whereas transgenic

overexpression of leptin in osteoblasts has no effect on bone mass. These data suggest that leptin decreases bone mass indirectly via activation of the SNS (185). However, a recent paper describes a leptin osteogenic direct effect on mouse long bone (689) in line with data supporting osteoblast proliferation and mineralization (347,899). Therefore, leptin has several different endocrine functions in addition to its effects on energy homeostasis.

BAT activation and browning reduce the leptin plasma levels (with pushing effect to food search and intake) in line with the need to maintain an equilibrium between BAT and WAT in the adipose organ (707). Of note, classic brown adipocytes do not produce leptin (177), but intermediate forms during whitening of brown adipocytes may produce leptin in line with data suggesting a reciprocal regulation of leptin and UCP1 genes in adipocytes (107).

Leptin is also produced by salivary glands (228) and stomach (27,174,178). An inhibitory activity of leptin on the orexigenic ghrelin (hormone also produced by stomach) has been shown (443), thus a possible role of leptin in the physiologic mechanism of interruption of food intake cannot be excluded.

Leptin receptors have been shown also to be present in preadipocytes and leptin induces adipogenesis *in vitro* (553, 952). Interestingly db/db mice (lacking ObR) is the only model of genetic obesity in which adipose tissue is only hypertrophic (433), thus leptin (that is secreted in positive relationship with the size of adipocytes) con be involved in the signaling ending with the stimulus for neo synthesis or development of fat cells (66,386).

In this contest the mammalian target of rapamycin Complex 1 (mTORC1) (790,791) and its effector, ribosomal protein S6 kinase 1 (S6K1) seem to play an important role because mice lacking S6K1 have normal adipocyte terminal differentiation but altered adipogenesis (119). Furthermore, mice with an adipose-specific deletion of Raptor (regulatory-associated protein of mTORC1), which is required for normal mTORC1 activity, have a similar phenotype to that of the S6K1 knockout mice (696). A recent paper confirms the importance of mTOR as a critical regulator of adipogenesis (816).

Adiponectin It is an approximately 30-KDa protein exclusively produced by adipocytes and is abundant in the plasma (315, 410, 556, 798). It is more expressed in subcutaneous than in visceral fat both in white and brown adipocytes (420,945,1012).

The adiponectin production seems to follow an opposite rule respect that of leptin: there is a strong negative correlation between plasma concentration of adiponectin and fat mass (410). Adiponectin circulates in serum as a range of multimers from trimers to high-molecular-weight (HMW) dodecamers (39). The HMW adiponectin seems to be largely the most important form accounting for most of its peripheral effects with the exclusion of CNS where low-molecular-weight trimers and hexamers are prevalent. This hormone improves whole-body insulin sensitivity probably acting on AMP activated kinase (AMPK) of muscle cells by a signaling through the adiponectin receptor 1 (AdipoR1) (195,665,803,961,962,986,987). Another important role on glucose metabolism seems to be the suppression of hepatic glucose output through the AMPK activation (195,988). An orally active adiponectin receptor agonist has been shown to improve insulin sensitivity and lifespan in genetically diabetic obese mice (656).

Within the vascular wall, adiponectin exerts its anti-atherosclerotic activity by inhibiting monocyte adhesion, macrophage transformation to foam cells and decreases proliferation of migrating smooth muscle cells in response to growth factors (571,1011).

In addition, adiponectin stimulates nitric oxide production in endothelial cells and promote angiogenesis (135). These effects are mediated via increased phosphorylation of the insulin receptor, activation of AMPK, and modulation of the nuclear factor nkB pathway (132,244).

Adiponectin receptors are also present in the central nervous system and low level of adiponectin are present in the cerebrospinal fluid and here adiponectin (low-molecular-weight) stimulates appetite and reduce energy expenditure acting on AMPK (481). AdipoR1 and 2 colocalize in hypothalamus with leptin Ob-RL receptors and have been suggested that the central actions of leptin and adiponectin have reciprocal functions to provide a homeostatic mechanism to maintain fat levels thought the regulation of appetite and energy expenditure (709).

Resistin It is an approximately 12-KDa polypeptide produced mainly by white adipocytes with a 15 times higher expression in visceral than in subcutaneous fat (31, 869). Resistin seems to play a role in glucose homeostasis of mice probably mediated by a mechanism involving the activation of SOCS3 (suppressor of cytokine signaling 3), an inhibitor of insulin signaling and by decreased activity of AMPK and reduced expression of gluconeogenic enzymes in the liver. In humans, Resistin is mainly produced by macrophages and obese fat is infiltrated by these cells (see obese adipose organ paragraph). Its physiologic role is still poorly understood even if several evidences point toward its potential link between obesity-inflammation and metabolic diseases (807). Recent data suggest also a role for resistin in the progression of human breast cancers in obesity and *in vitro* studies seems to support a link between resistin and ERM proteins (Ezrin, Radixin, and Moesin family that links F-actin to cell membrane proteins). ERM proteins may have an important role in tumorigenesis, cancer cell invasion, cross-cell signaling, and tumor metastasis, possibly via regulation of adhesion molecules (501).

Asprosin It is a 140 amino-acid secreted polypeptide abundantly expressed by mature white adipocytes of humans and rodents. Circulating levels of asprosin increase in fasting conditions and the hormone acts on the liver stimulating glucose production via cAMP-PKA signaling pathway. In obese humans and mice, plasma levels of asprosin increase in

parallel with insulin and could play a role in the metabolic syndrome because blocking its action a reduction of insulin and glucose hepatic production was observed. Of note, its absence in patients with neonatal progeroid syndrome (partial lipodystrophy that allowed Chopra and colleagues to discover this adipokine) could explain the absence of metabolic syndrome in these patients. Asprosin crosses the blood-brain barrier and directly activates orexigenic neurons. In patient with neonatal progeroid syndrome, its absence causes low appetite and extreme leanness (254, 751).

C3, Factor B, and Adipsin These are proteins of the alternate complement system all synthesized and secreted by adipocytes (977). The C3-derived ASP (acylation-stimulating protein) promotes lipoprotein lipase (IPL) activity and triglycerides synthesis and decrease lipolysis and release of fatty acids from adipocytes. ASP also increases glucose transport by translocation of glucose transporters. Both ASP and Adipsin enhance glucose-stimulated insulin secretion from pancreatic β-cells (151,532,976). In particular, C3a, a peptide generated by adipsin, is a potent insulin secretagogue and its C3a receptor is required for the beneficial effects of adipsin. Thus, the adipsin/C3a pathway seems to connect adipocyte function to β-cell physiology, and offer an explanation to the link between lipoatrophy and diabetes (see lipoatrophy paragraph).

PAI-1 Plasminogen activator inhibitor 1 (PAI-1) is a major regulator of the fibrinolytic system that is the natural defense against thrombosis. This serine protease inhibitor is produced also by liver, vessels, and platelets. It is mainly produced by visceral fat and TNFα seems to play a role in its regulation, thus suggesting a link between visceral obesity inflammation and risk of thrombosis (437, 846, 953).

Apelin It is a bioactive peptide known as the ligand of the G protein-coupled receptor APJ. Originated from a common 77-amino-acid precursor, three active apelin peptides exist under the form of 13, 17, or 36 amino acids. Apelin and APJ mRNA are widely expressed in several tissues of humans and rodents including stomach, heart, skeletal muscle, and WAT. It has functional effects in both the central nervous system and peripheral tissues (124). Apelin has been shown to be involved in the regulation of cardiovascular functions, fluid homeostasis, vessel formation, and cell proliferation. Apelin has been described also as an adipocyte produced and secreted factor, upregulated in obesity. Expression of apelin gene in adipose tissue is increased by insulin and TNFα. Positive as well negative effects on rodent glucose and hepatic metabolism has been described. Higher apelin serum levels have been found in obesity, (124, 478).

Apelin receptor antagonist treatment of rats showed diminished hepatic fibrosis (703).

Daily i.p. apelin injection was shown to decrease the triglycerides content in adipose tissue and the weight of different fat depots (393). Plasma triglycerides were also decreased in both normal and obese apelin-treated mice. The treatment did not affect average food intake but increase circulating levels of adiponectin, reduced leptinemia and increased rectal temperature and O_2 consumption, thus suggesting a browning effect. These data are in line with the observed increased expression of mitochondrial UCP1 in BAT (393) and with the claimed positive effects of browning and negative effects of whitening of adipose organ (897).

Omentin It is produced by nonadipocyte cells in adipose depots, it is mainly found in visceral adipose tissue rather than in subcutaneous adipose tissue (992). Plasma omentin levels are reduced in obesity, insulin resistance, and type 2 diabetes. Omentin has insulin-sensitizing effects and also has been reported to have anti-inflammatory, antiatherogenic, and anticardiovascular disease properties (887).

Retinol-binding protein 4 (RBP4) Retinol-binding protein 4 (RBP4) is a protein mainly produced by visceral adipocytes and hepatocytes (467). Its physiologic role is related to binding and transport of retinol, but other roles have been suggested mainly in relationship to its elevated levels in obese and insulin resistant humans and mice. A recent paper, using mice with hepatocyte-specific deletion of RBP4 (LRKO) showed that adipose tissue does not contribute significantly to circulating RBP4 even in presence of an expected increase during diet-induced insulin resistance. The authors conclude that adipocyte RBP4 is not a significant source of circulating RBP4, even in the setting of insulin resistance. Adipocyte RBP4, therefore, may have a more important autocrine or paracrine function that is confined within the adipose tissue compartment. In line with this idea, it has been shown that RBP4 is able to activate adipose tissue antigen-presenting cells (59,597) with consequent activation of innate immunity and promotion of adipose tissue inflammation, thus contributing to the molecular link between adipose tissue inflammation and insulin resistance (351,475,991). Activated BAT also release RBP4 (755) but its functional role is uncertain and should not be related to insulin resistance because it is well known that BAT activation favors insulin sensitization (41).

Vaspin Visceral adipose tissue-derived serpin (Vaspin) is a member of serine protease inhibitor family. Its expression has been found in human adipose tissue, stomach, liver, pancreas as well as in hypothalamus of db/db and B6 mice. Lean human individuals have undetectable vaspin mRNA in fat, whereas its expression increase in overweight and obese individuals especially in visceral fat (78, 392). This was not confirmed in all work (500). Administration of recombinant vaspin to obese mice improves glucose tolerance and insulin sensitivity and acutely reduces food intake. In addition, antiapoptotic effects of vaspin have been described in endothelial cells.

The mechanism of action is not known but it has been proposed that vaspin glucose lowering effects are due to its

serpin inhibition of the protease kallikrein 7, which plays a role in the half-life of insulin (389).

Visfatin In 2005, Fukuara et al. (312) proposed a new adipokine mainly secreted by visceral fat with insulin-like properties, but 2 years later, the authors retracted the paper for lack of reproducibility of the hypoglycemic properties and subsequent studies showed that visfatin is identical to pre-B cell colony-enhancing factor (PBEF), a previously described cytokine promoting maturation on early B-lineage precursor cells (781), but the name remain widely used. Visfatin displays intrinsic enzymatic activity as a nicotinamide phosphoribosyltransferase (Nampt), and is now better referred as Visfatin/Nampt/PBEF (749, 750, 752). Some reports deny a specific production by visceral fat (55, 466, 888), but an increased visfatin gene expression in visceral fat has been found in obese patients (667) and participation in inflammatory mechanisms of human visceral fat has been suggested (81). Visfatin is produced also by other organs including liver and skeletal muscles (308, 318, 321, 484), but also macrophages (that infiltrate the fat of obese animals and patients, see obese organ paragraph) are a physiologic source of visfatin (207, 349, 651). Visceral fat is always in tight anatomical relationship to vessels and one of the main activity of visfatin could be related to cells of vascular walls including characteristic macrophages of atherosclerotic plaques (749, 750).

Nesfatin-1 Originally described as a satiety molecule in the hypothalamus (652), it was subsequently found in several peripheral tissues including fat. This peptide derives from a precursor molecule (nucleobinding2: NUCB2) by prohormone convertase that produces three cleavage products: nesfatin 1, 2, and 3. In fat, it is mainly produced by subcutaneous adipose tissue both in humans and mice and its synthesis and secretion increase with adipocyte differentiation. Similarly, to leptin its expression also increases in high fat diet and decrease during fasting (716). Interestingly both in murine and human stomach nesfatin-1 is colocalized with ghrelin in endocrine cells. Thus, the same cell can secrete molecules with opposite effects on food intake (hunger for ghrelin and satiety for nesfatin-1) (868). Several other peripheral tissues also produce nesfain-1 including pancreatic β-cells, heart, anterior pituitary gland and testis, suggesting its involvement in several homeostatic pathways including a potentiation effect on glucose-induced insulin secretion on pancreatic β-cells (620, 718).

DPP-4 DPP-4 (dipeptidyl peptidase IV) is a protein that is expressed by endothelial cells, salivary gland, prostate, seminal vesicles, endometrium, renal tubules, and small intestine and decidual cells (278, 311, 609). One physiologic role of this enzyme is to degrade incretins and DPP-4 inhibitors are in clinical use as antidiabetic drugs (105). Human adipose tissue was shown to be an additional source of circulating DPP-4. Its serum concentration correlate with adipocyte size, and visceral fat of obese patients showed a fivefold higher level of protein expression than subcutaneous fat but no difference was found in lean subjects. Its secretion in visceral obese subjects could therefore contribute to development of metabolic syndrome (216, 490, 511, 748).

Cannabinoids Endocannabinoids (ECs) are lipids with autocrine and paracrine actions (668, 838). They are synthesized in the cells on demand from cell membrane phospholipids and immediately released to target their receptors (CB1 and CB2) that are usually localized in the neighbor cells or in the same EC producing cell. The most studied are N-arachidonoylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG). CB1 receptor is mainly expressed in the central nervous system and mainly in the areas dealing with the energy intake. In particular, neurons responding to the main peripheral satiety and orexigenic hormones use cannabinoid as modulators of their activity. Here they function in a retrograde manner: they are produced by postsynaptic cells and act on CB1 in presynaptic terminals to inhibit excitatory or inhibitory neurotransmitter release (654). Endocannabinoid release is immediate without any storage in vesicle allowing a real-time answer to the variable feeding state of the organism. Furthermore, they have an important role as modulators of neurons in the mesolimbic system, thus participating in both the homeostatic and hedonic aspects of food intake (98, 242, 866). It has also been shown an influence of EC on neural circuits involved in the control of energy dissipation (439, 713).

In peripheral tissues, CB1 is expressed mainly in organs, which play important roles in metabolic homeostasis. CB2 is mainly expressed in cells of the immune system (409).

Mature white adipocytes express CB1 and enzymes for the production and degradation of EC (52, 79, 200, 570, 744). In white adipocytes, CB1 activity seems to stimulate the processes of lipogenesis and inhibit lipolysis (241, 839, 944). Most of these data come from *in vitro* studies, but it has been also shown that treatment of baboons with the CB1 antagonist rimonabant induces a weight-loss independent healthy activity on adipose tissues (930).

Brown adipocytes also have functional CB1 receptors, but their functional role remain to be elucidated although some data point to their inhibition of sympathetic inputs to BAT and consequent decrease of thermogenesis and whitening of adipose organ (30, 713, 838).

Very recently, Ruiz de Agua et al. showed that mice lacking CB1 specifically in the adipose organ show a browning of visceral and subcutaneous fat with increase of parenchymal noradrenergic nerve fibers density and a tight anatomical relationship between M2 macrophages and nerve fibers (770).

Growth factors (FGF21, BMPs, TGFβ, and GDFs)

FGF21. It is a growth factor without relevant proliferative mitogen capacities (344, 633, 636, 900, 974). It is mainly expressed by liver, but after cold stimulus it is also produced and secreted in the blood by BAT, whereas in this

condition liver production decrease (947). This adipokine has important browning properties both direct on WAT and indirect through increase of sympathetic outflow. It is secreted both in mice and humans also by adipocytes with intermediate phenotype between white and brown (i.e., paucilocular adipocytes also known as beige/brite), thus reinforcing the WAT browning phenomenon induced in WAT after cold exposure (291,402,403,504) (see browning paragraphs). Furthermore, FGF21 induces glucose oxidation in many tissues thus promoting protection against obesity and T2 diabetes (199, 344).

BMPs. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β superfamily. They are morphogens (62,717,925) that play an important role in the development of many tissues including adipose tissues. As morphogens, they act in the site where they are produced. The adipose organ is highly plastic and its development and involution can be required during the whole lifespan of mammals, thus it is not surprising that morphogens play an important role in its development and remodeling plasticity.

BMP2 has been claimed to play a role in white adipocyte differentiation (593, 1003) as a matter of fact mice lacking Schnurri-2 (a downstream regulator of the BMP-2 signaling pathway) have a drastic reduction of WAT (431). BMP7 seems to play a key role in brown adipocyte development. In fact, BAT development is impaired in mice lacking BMP-7 (593,710,922). BMP4 activity is not well elucidated because data support its role in white adipocyte development, but its transgenic expression induces WAT browning and protect from diet-induced obesity (710, 913). TGFβ and activin A inhibit differentiation of both white and brown adipocytes (999), but mice lacking Smad3 (downstream) signaling show WAT browning with all its healthy consequences (985). The activity of BMPs seems to derive from a balance with inhibitors such as TGFβ, activin A and Gremlin1 (370). Most BMP signaling is mediated via two receptors: type 1 (BMPR1) and/or type 2 (BMPR2) and downstream activation of the SMAD transcription factors (593,710). Interestingly, specific genotypes of the BMPR isoforms BMPR1A and BMPR2 have been shown to associate with obesity in human (87,134,800). Of note, specific deletion of BMPR1a in Myf5-expressing cells (precursors of classic brown adipocytes) results in a severe paucity of BAT and the compensatory activation of WAT browning (805). Within adipose tissues, BMP7 is probably produced by the adipose tissue niche contained in the stromal vascular cells (593, 806). Thus, it is not surprising that the BMPs production seems to play an important role in commitment and determination of adipocyte progenitors (see also origin paragraph). BMP8b is mainly produced by mature brown adipocytes and physiologic stimuli (cold, high-fat diet) increase its expression (966). BMP8b functions locally by enhancing the response of BAT to β3-adrenergic stimulation. Its expression is gender specific (higher in females) and together with its local activity BMP8b seems to activate BAT also through the sympathetic nervous system. GDF5 (growth differentiation factor5) (399) and BMP9 (485) are other BMP/TGFβ members that seem to induce WAT browning. It has been also shown that BAT is able to produce GDF8 (growth differentiation factor 8 or myostatin) with inhibitory autocrine effects (95,867) and conversely follistatin with positive excitatory autocrine effects (843).

Vasculotrophic factors (VEGF-NO-CO) The adipose organ plasticity regarding browning and nerve-vascular remodeling seems to be strictly connected to the ability of adipocytes to synthesize and secrete vascular and neuroregulatory factors.

VEGF. The vascular endothelial growth factor (VEGF) family is composed of six secreted glycoproteins: VEGF-A, B, C, D, E, and placental growth factor (PlGF) (664).

VEGF-A (VEGF) is one of the most potent angiogenic factors (635) and is expressed both in white and brown adipocytes *in vivo* and *in vitro* (24,182,592). Our data showed that VEGF production appears to be under the stimulatory control of noradrenaline, mainly through β3-adrenoceptors, thus allowing the supply of VEGF when functionally required and explains its deficit in genetically obese animals (904).

VEGF is very important for normal embryonic development because experimental deletion of even a single VEGF allele results in abnormal blood vessel development and embryonic lethality by E9.5 in murine models (118, 287).

VEGF ligands bind specifically to two receptor tyrosine kinase membrane-bound proteins—VEGFR1 and VEGFR2 (considered primary signaling receptor), which are expressed in most endothelial cells (310).

Antisense knockdown of VEGFR1 does not affect endothelial cell proliferation, migration, and platelet activating factor expression, while knockdown of VEGFR2 severely impairs these processes (54). Furthermore, VEGF seems to have together with paracrine activities on endothelial cells also autocrine properties because induces the master regulator of mitochondrial biogenesis: PGC-1α, and have direct beneficial effects on brown adipocytes to promote their survival, proliferation, and maintenance of mitochondria (28). In line with these data, cold exposure induces browning and vasculogenesis with upregulation of VEGF in inguinal WAT that is hypoxia independent and VEGFR2 dependent (984).

NO. White and brown adipocytes express different isoforms of nitric oxide synthase (NOS) and thus synthesize and release nitric oxide (NO) via noradrenergic stimulation (266, 616, 642). NO seems to play a role in the sympathetic induction of BAT vasodilation to match thermogenesis with perfusion, as well as in the proliferation and differentiation of brown adipocytes *in vitro* (341).

The main function of NO seems to be promotion of mitochondria biogenesis and bioenergetics with favorable impact in several chronic diseases including obesity, T2 diabetes (638, 931).

CO. We also showed that brown adipocytes express the isoenzymes for the production of heme oxygenase (HO). HO is a ubiquitous microsomal enzyme, which produces a gaseous mediator, carbon monoxide (CO), and plays a crucial role

in maintaining cellular heme homeostasis and hemoprotein levels (559, 560).

Interestingly their localization in the adipose organ was found in the cytoplasm and nuclei of brown adipocytes and in vascular walls. In brown adipocytes, the cold exposure upregulated the HO-1 isoform suggesting that the HO system may be involved in brown fat function (337).

Angiotensin. The renin–angiotensin system (RAS) is a well-known system that play a key role in the regulation of blood pressure. Angiotensinogen is cleaved by the enzymes renin and angiotensin-converting enzyme, to form angiotensin II that is the main bioactive peptide of this system. Angiotensin II exerts its physiological actions, primarily via two G-protein coupled receptors: Ang II type 1 (AT1R) and type 2 (AT2R) receptors (801). Both WAT and BAT are sites for the production of the major components of RAS and AT1 and 2Rs (123, 267, 569). Transgenic mice overexpressing angiotensinogen in adipose organ (driven by aP2) develop hypertension, white adipocyte hypertrophy and insulin resistance (442, 567, 568).

Neurotrophic factors (NGF, Semaphorins, and Nrg4) The very important role of parenchymal noradrenergic fibers in the activation of BAT and browning of the organ is described in paragraph on noradrenergic parenchymal nerve fibers plasticity. Two neurotrophic factors (NGF and Sema3) seems to counteract and one (Ngr4) promote the parenchymal innervation of adipose organ.

NGF. The potent neurotrophic factor NGF that promotes the survival and proliferation of neurons (288, 515) is produced *in vivo* and *in vitro* also by white and brown adipocytes (624, 641, 682, 855). Its production seems to be in an inverse correlation with BAT functionality, thus suggesting a role restricted to the maintenance of existing innervation (643).

Semaphorins. Murine BAT express Sema3a, a chemorepellent neuronal factor active on both sympathetic and sensory peripheral nerves (470). In rats maintained in thermoneutral conditions, brown adipocytes produce both active isoforms of Sema3a and show a distinct peripheral polarized immunostaining pattern suggesting a role for Sema3a secreted by brown adipocytes in the guidance of axons growth (329, 330). In cold-acclimated rats, where parenchymal nerve fibers density is higher, both the expression and the immunostaining of the two active isoforms are reduced.

Thus, Sema3a could play a role in the plastic adjustment of BAT innervation observed in different conditions of functional request.

A recent study confirms the importance of semaphorins system in BAT and showed that M2 macrophages lacking the nuclear transcription regulator Mecp2 ('methyl-CpG-binding protein 2') increased the expression of PlexinA4 that might act to repel Sema6A expressing sympathetic axons in BAT and thereby diminish its innervation (969).

Nrg4. Nrg4 (neuregulin 4) is produced and secreted by mature adipocytes *in vitro* and *in vivo* and induces nerve growth *in vitro* in a dose dependent manner (687, 756, 957). During cold exposure, murine WAT expresses more Nrg4 than BAT supporting the idea that this adipokine play a key role in the growth of peripheral sympathetic nerve fibers, thus playing a key role of adipose organ browning (148).

Inflammatory cytokines (TNFa, IL6, IL33, IL1B, RANTES, IL-8, SDF-1, MIF, and MCP1) A series of proinflammatory citokines are also produced by white adipocytes as well as by other cell types (mainly: macrophages, eosinophils and lymphocytes, see also immunebrown paragraph) of WAT.

The most studied is TNFα because of its high expression in obese fat and its property to interfere with insulin signaling by reducing tyrosine phosphorylation on insulin receptor (IR) and insulin receptor substrate 1 (IRS1) on key tissues: skeletal muscles, liver and fat (405, 407, 858, 861, 980). Two papers in 2003 (964,979) showed that the increased expression of TNFα in obese fat was mainly due to macrophages (see obesity paragraph), thus the role of TNFα secreted by adipocytes remain to be established. Interestingly *in vitro* studies showed that TNFα is able to increase the secretion of IL-33 by adipocytes and IL-33 could play a role in the modulation of activities of innate immune cells of WAT (970).

Many other cytokines are produced by adipocytes and their roles on different types of leucocytes normally present in the adipose organ are at beginning of their exploration. Most of them (IL-6, IL-1B, RANTES, IL-8, IP-10, SDF-1, MIF, and MCP1) have chemotactic properties (343, 446) suggesting a possible physiologic functional interrelationship, but their precise functional role to date is far to be known. It must be remembered that some depots of the adipose organ are particularly rich in leucocytes (omentum and mesenteric fat) suggesting a more specific functional relationship for those depots (796).

Human BAT-like tissue derived from the capillaries of subcutaneous WAT and implanted into mice with diet-induced obesity improved the metabolic syndrome and produced secretory factors: IL-33, proprotein convertase subtilisin/kexin type 1 (PC1/3), proenkefalin (PENK) (591).

MCP1 is a potent chemoattractant that play a key role in macrophages infiltration of obese fat (29, 711, 721) (see obesity paragraph).

Lipid metabolism (LPL, CETP) LPL is a key regulator of triglycerides deposition in adipocytes. It is synthesized by both visceral and subcutaneous adipocytes and transferred to the luminal surface of endothelial cells by transcytosis. Insulin and glucocorticoids are the physiological stimulators of LPL. This enzyme hydrolyzes the triglyceride in circulating lipoproteins such as chylomicrons and VLDL (very low-density lipoprotein) and produces free fatty acids that are used for metabolic energy or for fat storage (17).

Colesteryl-ester protein transporter (CETP) is mainly produced by visceral adipose tissue in humans (314). This protein promotes the remodeling of plasma lipoproteins,

thus influencing their peripheral metabolism (886). CETP mainly promotes the exchange of cholesterol esters and triglycerides between plasma lipoproteins. It appears to be an important actor of the so-called reverse cholesterol transport from peripheral tissues to the liver and excretion (728, 831, 886, 953).

Zinc-alfa2-glycoprotein (ZAG) is a lipid-mobilizing factor expressed by white adipocytes that could play a role in cachexia (63).

Other BAT-adipokines (batokines) M20 domain-containing protein 1 (PM20D1) is a secreted enzyme, highly enriched in brown adipocytes. It catalyzes the synthesis of N-acyl amino acids from free fatty acids and the reverse hydrolytic reaction. N-acyl amino acids directly bind mitochondria and function as endogenous uncouplers of UCP1-independent respiration increasing whole body energy expenditure (538).

The C-terminal fragment of secreted Slit2: Slit2-C promotes thermogenesis activating the PKA signaling pathway (883).

Other endocrine effects (T4/T3, cortisone/cortisol, and androgens/estrogens) **T4/T3.** BAT produces type II thyroxine 5′-deiodinase (Dio2), which converts T4 (thyroxine) to T3. It is highly induced during BAT activation (837, 956). It has been suggested that BAT-derived T3 is important as a local stimulus for thermogenesis because mice lacking Dio2 suffered hypothermia upon cold exposure despite normal plasma T3 levels (219).

Cortisone/cortisol. Cortisol (corticosterone in rodents) availability and action depend not only upon circulating levels and its ability to bind and activate the glucocorticoid receptor (GR), but also by the activity of two isoenzymes: 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2) (598). 11β-HSD2 inactivates cortisol and it is highly expressed in mineralocorticoid target tissues [salivary gland, kidney, and colon (599)] to prevent the activity of cortisol on the mineralocorticoid receptor (MR).

11β-HSD1 have the opposite function and is mainly expressed in highly metabolic tissues such as liver, fat, and skeletal muscles. Clinical data from patients with excess of circulating cortisol deriving from adrenal or pituitary tumors with specific defects in the activity of 11β-HSD1 lacked the classic Cushingoid syndrome: visceral obesity, hypertension, skeletal muscle myopathy, insulin resistance, and T2 diabetes. Gain and loss of functions in total or tissue specific murine models have confirmed that tissue intrinsic 11β-HSD1 activity is the major determinant of the adverse metabolic manifestations of circulatory cortisol excess (598).

Interestingly, it has been shown that inflammatory cytokines (TNFa) are able to increase the activity of HSD1 (271) thus offering an explanation for a possible vicious circle in visceral fat where the fragility of adipocytes due to scarce expansibility can cause inflammation (see obesity paragraph). In this context, MR antagonism has been shown to protect mice from the adverse obesogenic and metabolic effects of a high-fat diet via conversion of a substantial amount of visceral and subcutaneous WAT into BAT (15, 692).

Androgens/Estrogens. Another important effect of adipose organ activity on circulating hormones is due to the enzyme cytochrome P450 aromatase (435, 671, 841).

WAT is an important site of aromatase activity that converts androstenedione and testosterone to estrone and estradiol. It has been calculated that about 80% of estradiol in men is produced in extragonadal tissues (551). Estrogen receptors are widely diffused in the organism and their functional role is much wider than that on female sex characteristics and reproductive capabilities (729, 840). In particular, estrogens have important effects on brain neurons plasticity (590), neuroprotection (562), and brain mitochondrial functionality (993). Furthermore, estrogens exert positive effects also in the bioenergetics system of the brain (97, 247) and in virtually all other peripheral organs (459, 729).

Distribution and activity of the two estrogen receptors α and β in adipocytes of the subcutaneous and visceral compartments account for the different sex-related distribution of fat in the adipose organ of women and men (824, 825) (see human adipose organ remodeling paragraph).

In synthesis, many endocrine-paracrine molecules produced by adipose organ play a role in its plasticity: neurotrophic factors and vasculotrophic factors influence the remodeling in nerve and vascular composition of the organ. All growth factors described, cannabinoids and apelin influence directly or indirectly the reciprocal WAT-BAT conversion and many (leptin, resistin, C3, PAI-1, adipsin, visfatin/Nampt/PBEF, Nesfatin-1, and DPP-4) are influenced by the conversion both for the change in parenchymal tissue composition or for the infiltration of inflammatory cells (mainly macrophages). Finally, also the influence of adipose organ on the endocrine system is also highly dependent from its cellular composition.

Lactation: Adipose tissue remodeling to milk producing glands

The plastic properties of adipose organ offer an explanation to its mixed anatomy: that is, in normal conditions energy derived from food intake is channeled toward the two main needs to survive: thermonesesis (BAT) and metabolism (WAT). In particular situations such as chronic cold exposure, it is necessary to increase thermogenesis (and plasticity of the organ allows the browning phenomenon) or increase the energy storing for metabolism such as in positive energy balance situations (and plasticity of the organ allows the whitening phenomenon). Thus, plasticity of the organ allows to adapt the organ function to specific needs. The cellular phenomenon at the base of this plasticity is widely accepted as due, at least in part, to a direct conversion (transdifferentiation) of white adipocytes into brown adipocytes and vice versa (2, 41, 153, 158, 168). This phenomenon implies a

physiologic reversible reprogramming (519) of adult cell genome and deserve further examples.

We found a striking new example of physiologic reversible transdifferentiation in the female adipose organ during pregnancy, lactation, and postlactation periods.

Mammary glands anatomy in adult virgin mice is very simple: branched epithelial ducts infiltrate subcutaneous fat and end in a single nipple. Five bilateral nipples are present in the ventral surface of the mouse skin, the first three collect ducts infiltrating the whole anterior subcutaneous depot and the last two collect ducts infiltrating the whole posterior subcutaneous depot. Thus, the real glandular part of mammary glands: that is, milk-producing alveoli, is not present in virgin adult mice (631, 734). Alveologenesis is a hormone-induced phenomenon of pregnancy and lactation periods (408). During alveologenesis a progressive reduction in number of adipocytes parallel the alveolar development and no or very few residual adipocytes are present in lactating mammary glands (Fig. 15). Following the steps of mammary morphogenesis during pregnancy we noticed three interesting structural aspects regarding: 1-Transforming adipocytes 2-Early alveoli anatomy 3-Intermediate structures between transforming adipocytes and early alveoli (606).

1-Trasforming adipocytes. We noticed that at the apex period of alveolar formation (around day 18th of pregnancy in mice) several adipocytes assume a morphology never observed in other physiologic condition: compartmentalization of lipid droplets, development of organelles in a thickened cytoplasmic rim (stacked rough endoplasmic reticulum, peroxisomes hyperplasia, hypertrophic Golgi complex, mitochondria hypertrophy) and development of cytoplasmic projections. Of note, this unusual anatomy of adipocytes coincided with their atypical immunoreactivity for Perilipin2 (typical of mammary alveolar cells) while maintaining the typical immunoreactivity for Perilipin1 (typical of adipocytes) (705).

In line with the hypothesis that transforming adipocytes give rise to alveolar cells, and in line with the earlier reported ultrastructural findings in these adipocytes, it has been shown that mice lacking X-box-binding protein 1 (XBP1: a central

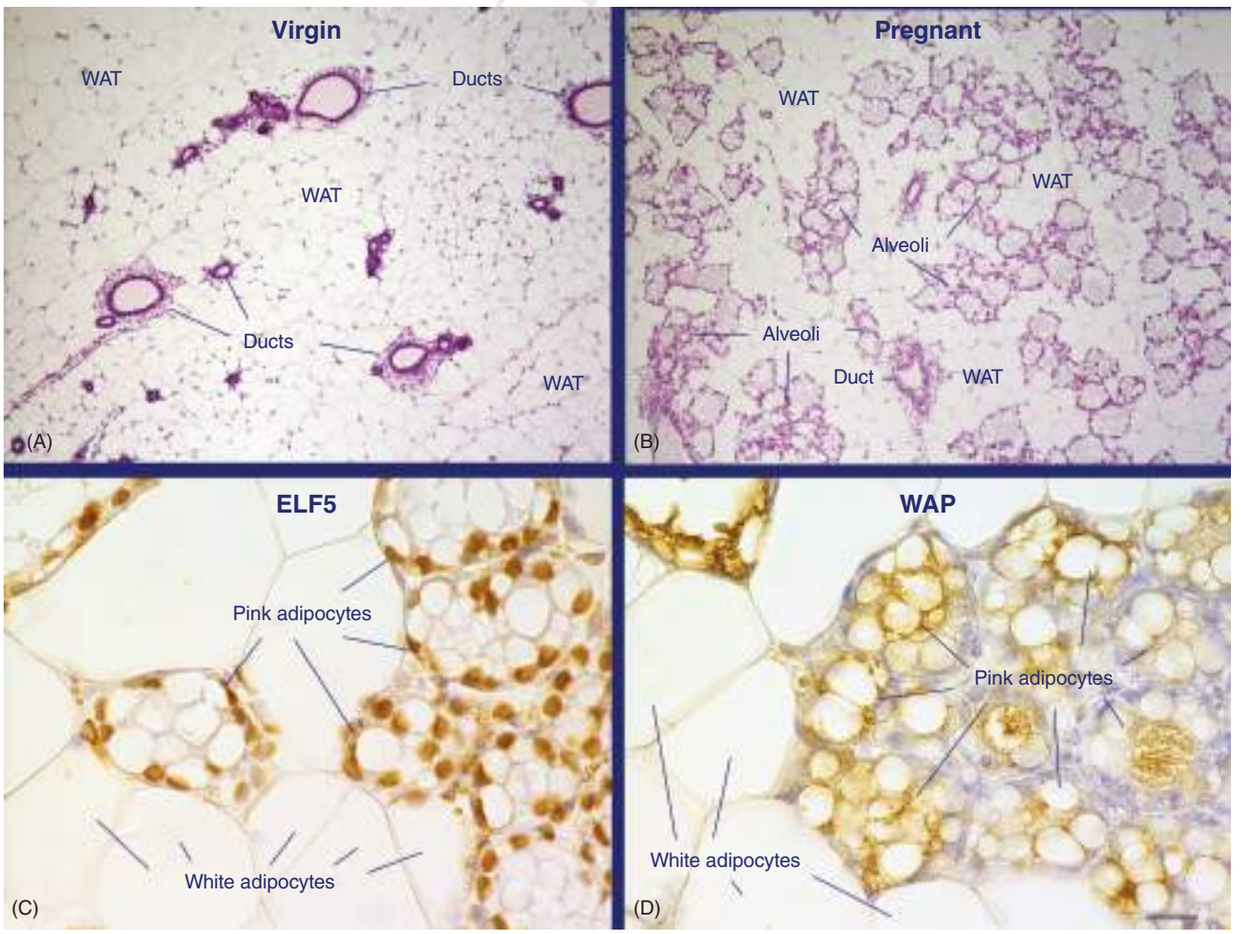

Figure 15 Histology of inguinal adipose tissue of virgin (A) and pregnant (B-D) mice. Epithelial alveolar cells appear only during pregnancy and are immunoreactive for ELF-5 (C) and WAP (D). This milk-producing alveolar cells show large cytoplasmic lipid vacuoles (pink adipocytes). Bar: in A and B, 50 μm; and in C and D, 12 μm. Adapted, with permission, from (167).

regulator of endoplasmic reticulum adaptive responses) specifically in fat had no effect on adipocyte formation but a blunted process of alveologenesis during pregnancy with decreased milk production (359). Furthermore, the absence of membrane channel Pxmp2 in peroxisomes of mammary fat altered the normal development of alveoli during pregnancy (941).

Moreover, we showed that together with alveolar cell nuclei only nuclei of some transforming adipocytes (and not adipocytes without signs of transformation) at day 18th of pregnancy resulted immunoreactive for ELF5 (E74-like factor 5, ets domain transcription factor) (705), that is a potent and specific transcription factor that is essential to induce alveologenesis (143, 493, 650).

2-Early alveoli (around day 18th of pregnancy, that is the time of major alveolar development) anatomy revealed a characteristic impressive abundance of cytoplasmic lipids in the epithelial cells (734). In fact, glandular epithelial cells (forming a well visible lumen, joined by typical epithelial junctions, with apical classic milk-protein secretory granules identical to those secreted in the lumen), immunoreactive in the cytoplasm for milk-typical whey acidic protein (WAP) and presenting ELF-5 immunoreactivity in the nuclei, contained a single cytoplasmic lipid droplet conferring to these cells a morphology more close to that of adipocytes than to any other glandular epithelial cell in the organism (734, 851). Our reasoning was that these elements are lipid-rich parenchymal cells of adipose organ, thus, by definition, they are adipocytes (considering that the term adipocyte imply only the abundant cytoplasmic lipids without any physiologic implication). Thus, we called these cells pink adipocytes because the color of the organ during pregnancy is pink (153, 333, 339). Interestingly, some alveolar structures develop also earlier (between 15th and 17th day of pregnancy) and most of them lack the cytoplasmic lipid droplets described earlier suggesting that these early alveolar structures could derive from ductal stem cells progenitors (851).

3-Intermediate structures with a morphology intermediate between transforming adipocytes and early alveoli are often found in this period of pregnancy (day 18th). They are multinucleated structures with typical features of very early alveoli (with milk-protein granules, nuclear immunoreactivity for ELF-5 and presence of myo-epithelial cells), but with cytoplasmic lipid droplets of the same size of that contained in surrounding adipocytes (606, 851).

These data suggest that under the hormonal pregnancy stimulus adipocytes of the mammary gland gradually transform their anatomy, aggregate with other adipocytes and myoepithelial cells to form intermediate structures and finally develop a glandular alveolar anatomy.

On the other hand, at the end of lactating period adipocytes reappear in the gland in parallel with a progressive disappearance of alveolar structures. In mice, the pre-pregnancy anatomy is reconstituted ten days after the end of lactation. We noticed that not all the alveolar epithelial cells underwent an apoptotic process and some of them accumulated lipid droplets with a progressive development into adipocytes. Of note, about 17% of postlactation developing adipocytes showed cytoplasmic granules similar to those typical of alveolar epithelial cells (606).

Thus, the complete picture suggested by our morphologic and immunohistochemistry analyses was a physiologic and reversible adipo-glandular transdifferentiation guided by pregnancy and lactation hormonal environment. This would strongly confirm the plastic properties of adipose organ, again for an energy partitioning, but this time not for animal survival but for species survival.

Lineage tracing experiments seems to be the ideal technique to demonstrate the direct conversion of a cell into another type of cell (854,911). Double transgenic mice able to express a cell specific and temporally specific reporter gene, that will be expressed thereafter whatever phenotypic conversion will happen, are also commercially available. We used aP2-Cre/R26R mice, that express beta-galactosidase (beta-gal that can be visualized by x-gal histochemistry) only in adipocytes to demonstrate adipo-epithelial conversion. In virgin mice only adipocytes and not ductal epithelial cells resulted x-gal positive. About 60% of alveolar cells were x-gal positive at day 18th of pregnancy, suggesting an important contribution of adipo-epithelial conversion in alveologenesis (606). We than used WAP-Cre/R26R mice that express beta-gal only in milk-producing epithelial cells to demonstrate the epithelial-adipo conversion. Data showed that in virgin mice neither adipocytes and epithelial cells were x-gal positive, during pregnancy only epithelial cells were positive and in the postlactation also adipocytes were positive in the first postlactation day, after 10 days and after 6 months (606). Furthermore, we found in the postlactation mammary glands, 10% to 15% of adipocytes immunoreactive for WAP a protein that is never expressed by adipocytes in virgin mice (705).

Recently, we also showed a gland-BAT (pink-brown) conversion in the postlactation period (338). In the dorsal part of the first three mammary glands interscapular BAT, that is functionally inhibited during pregnancy and lactation (479, 919), is in contact with glandular tissue and during pregnancy mammary glands infiltrate the peripheral part of interscapular BAT (Fig. 16). We noticed that in the first days of postlactation period some adipocytes with the ultrastructural features of classic brown adipocytes showed cytoplasmic structures with the classic features of milk-protein granules-containing vacuoles, usually found in milk-producing and secreting mammary alveolar cells. In WAP-Cre/R26R postlactating mice, we found beta-gal stained multilocular cells that resulted also marked by the specific immunostaining with UCP1 antibodies, thus confirming that alveolar epithelial glandular cells convert into thermogenic brown adipocytes in the postlactation period.

To confirm the striking adipo-epithelial (white-pink) conversion phenomenon, we explanted pure mammary fat from Rosa26 tagged mice (with all cells expressing beta-gal) into mammary gland of virgin wild-type mice. During pregnancy, explanted tagged fat gave rise to beta-gal positive alveolar

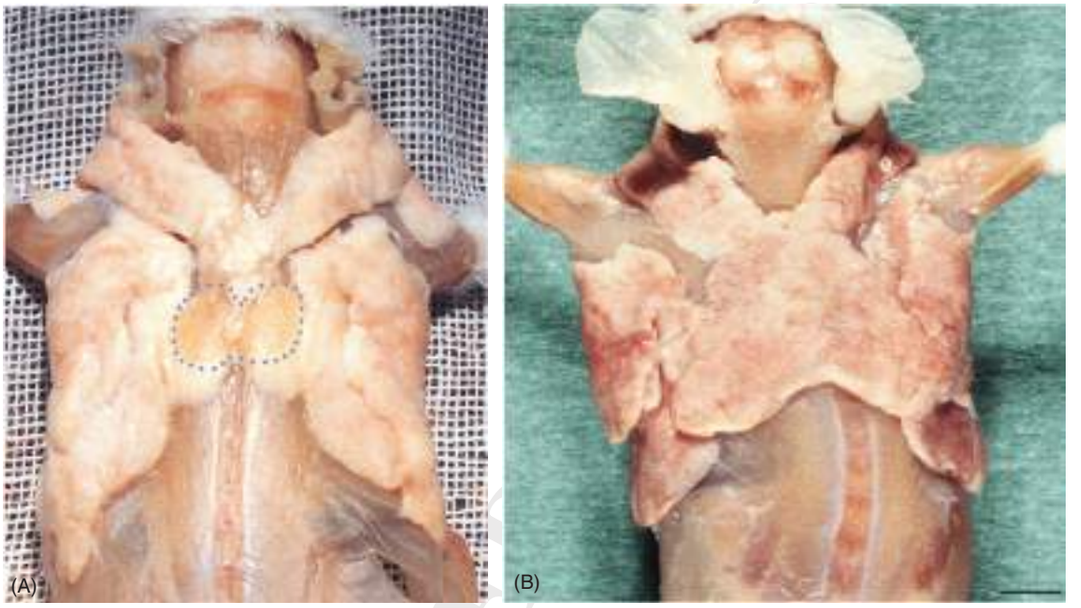

Figure 16 Dorsal view of anterior mammary glands. Note interscapular brown adipose tissue still visible in mouse in A and not visible in mouse in B. Bar: in A, 1.9 cm; and in B, 2.8 cm. Adapted, with permission, from (169).

structures among beta-gal negative native milk-producing glands of the host (230). Although the tagged glands derived from explanted fat resulted less developed than native glands they showed secretory products at electron microscopy that resulted milk-proteins immunoreactive at immunohistochemistry analyses. Furthermore, also explanted isolated mature adipocytes were able to differentiate into mammary glands during pregnancy (230).

Data showing brown to pink transdifferentiation are still lacking to complete the transdifferentiation triangle (Fig. 17).

Figure 17 The remodeling properties of adipocytes in the adipose organ. Adapted, with permission, from (169).

To dissect the molecular mechanisms responsible for the reversible adipo-glandular transdifferentiation we did a series of experiments. In the first experiment, we tested the effects of pregnancy on cleared fat pad. Since decades, it is possible to surgically remove the epithelial part of mammary glands leaving only the fat: cleared fat pad (234). Pregnant mice with cleared fat pad on one side and normal glands in the other side were analyzed by light microscopy that revealed no effects of pregnancy on cleared fat pad and suggesting a role for ductal produced and secreted paracrine factors. Wide gene expression analyses comparing clear fat pad and contralateral wild type glands at several time points of pregnancy showed that osteopontin could be one of the candidate paracrine factor (706). Of note, the osteopontin receptor is a beta3 integrin that is expressed in mammary adipocytes and differentially increased in the normal glands during pregnancy in parallel with the increased expression of osteopontin. Mice lacking osteopontin have impaired alveologenesis during pregnancy (630).

These data on subcutaneous fat-mammary glands strongly reinforce the concept of plasticity of adipose organ to partitionate energy between the short-term homeostasis (thermogenesis-browning and metabolism-whitening) and long-term homeostasis (lactation-pinking).

Interestingly and in line with the above data on adipo-mammary plasticity, very recently Li et al. showed that brown adipocytes can display a mammary basal myoepithelial phenotype).

The rainbow adipocyte

Since decades, it is widely accepted that mature adipocytes are able to dedifferentiate *in vitro* (875, 893). Considering the plastic properties of adipose cells observed *in vivo,* we wanted to further analyze the details of the plastic properties of mature adipocytes *in vitro*.

In 1986, Sughiara et al. developed a method to maintain mature adipocytes *in vitro* (876). They solved the problem due to the presence of abundant lipids in the cytoplasm of these cells. Lipids tend to float and push up the cells inducing detachment of cultivated cells from the bottom of culture system, thus interfering with their further maintenance-development. Thus, the solution was to reverse the vials to allow mature adipocytes to survive attached at the top of the vials: ceiling cultures.

This technique allowed to observe a progressive loss of lipids that was interpreted as a dedifferentiation-like phenomenon. The main structural characteristic of mature adipocytes consists in the cytoplasmic lipid droplet that occupy about 90% of the cell, thus the loss of lipid droplet transforms the adipocyte into a fibroblast-like cell similar, at light microscopy level, to poorly differentiated adipose cells (438, 875). Furthermore, delipidated (slimmed) fibroblast-like cells were considered de-differentiated also because they assumed gene expression profiles typical of mesenchymal stem cells (658, 699).

When adipocytes undergo a lipolysis process *in vivo*, such as during fasting, the morphologic modifications are very characteristic. In the early stages of lipolysis, when the lipid content is still almost intact, adipocytes produce cytoplasmic invaginations conferring a villous-like appearance at the cell surface (120, 169). These invaginations are very characteristic and allow distinguishing a slimmed adipocyte from any other cell type in the adipose tissue (Fig. 18). During lipolysis, the size of the lipid droplet reduces progressively and some smaller lipid droplets become visible at the periphery of the cell. In parallel with the size reduction of the lipid droplet, the cytoplasmic invaginations increase in size and number. Interestingly, the tannic acid method (76, 77) allows to see, by electron microscopy, the steps of this gradual slimming process because fatty acids are visualized as membranous lamellar whorls (LW) structures (75, 153). The LW structures are visualized at the lipid droplet surface, in the cytoplasm and specifically concentrate into the cytoplasmic invaginations (153). Here the LW structures seems to exit from the cells and fatty acids LW structures are visible in the interstitial space where they reach the capillaries (Fig. 19). Thus, this delipidated cell (slimmed) presents a quite characteristic morphology well far away from that typical of poorly differentiated mesenchymal stem cells. This delipidation process *in vivo* do not lead to a poorly differentiated phenotype and we observed that also *in vitro* delipidated mature adipocytes have a well differentiated phenotype, at electron microscopy level, well different form that typical for mesenchymal stem cells in spite of their expression of stem cell genes and in spite of their properties of multilineage differentiation (248, 286, 451, 572, 694, 698, 699, 820). Thus, we studied in details this spontaneous delipidation process of mature adipocytes *in vitro* and observed that it is completely different from that happening *in vivo* under fasting conditions. As a matter of fact, we showed ultrastructural (Fig. 20) and time lapse data (Fig. 21) supporting a process of liposecretion that allow a direct conversion of the mature adipocyte into a well differentiated postsecretion cell that we denominated rainbow adipocyte for its multilineage differentiation properties (575, 576). These data support the idea that also *in vitro* mature adipocytes are able of transdifferentiative capacities and maintain a unique plastic phenotype.

Pathologic Remodeling

Positive energy balance, aging and genetic alteration of adipocytes molecular signaling induce a peculiar remodeling of adipose organ with important unhealthy consequences. Some aspects of whitening remodeling are particularly important and offer an explanation to old clinical observations that can be viewed to date under a wider perspective including the plastic properties of adipose organ.

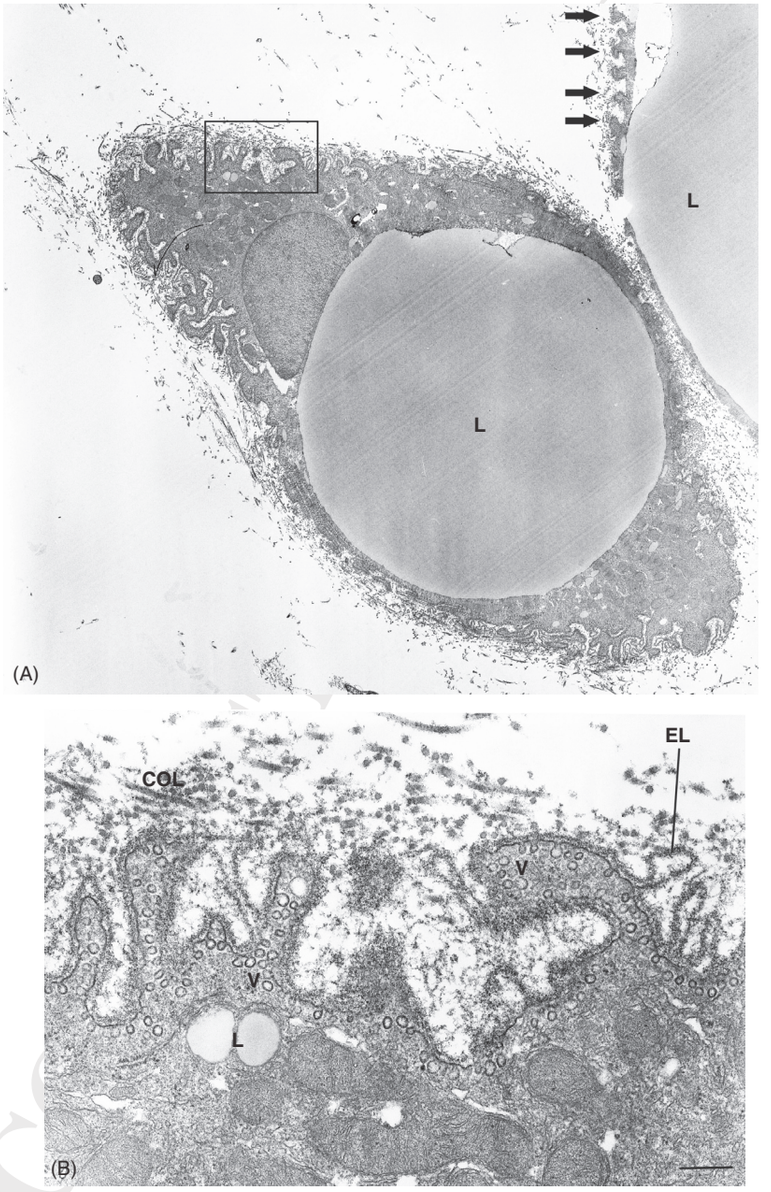

Figure 18 Electron microscopy of slimming adipocytes. Bar: in A, 1.6 μm; and in B, 0.2 μm.

The obese adipose organ

Hypertrophy and hyperplasia of adipocytes

In obese animals and humans, WAT of adipose organ is expanded due to an increased number of cells (hyperplasia) and to enlargement of single cell size (hypertrophy) (279, 280, 433, 574). The fat expansion in humans can reach 60% to 70% of total body weight (386, 704). Furthermore, most BAT in the adipose organ is transformed into a WAT-like tissue (whitening) especially in genetically obese mice (177).

In rare cases, the obese adipose organ is mainly or exclusively hyperplastic or hypertrophic.

Mice lacking the functional receptor of leptin (db/db) are massively obese and their WAT is exclusively hypertrophic (158, 433), suggesting a role for leptin in adipogenesis (see paragraph on origin of adipocytes).

Mice with "human-like" WAT (i.e., with high α2/β-AR balance in adipocytes) after high-fat diet (HFD) are obese with only WAT hyperplasia (932).

In all other known conditions of murine obesity, WAT is characterized by both hypertrophy and hyperplasia of

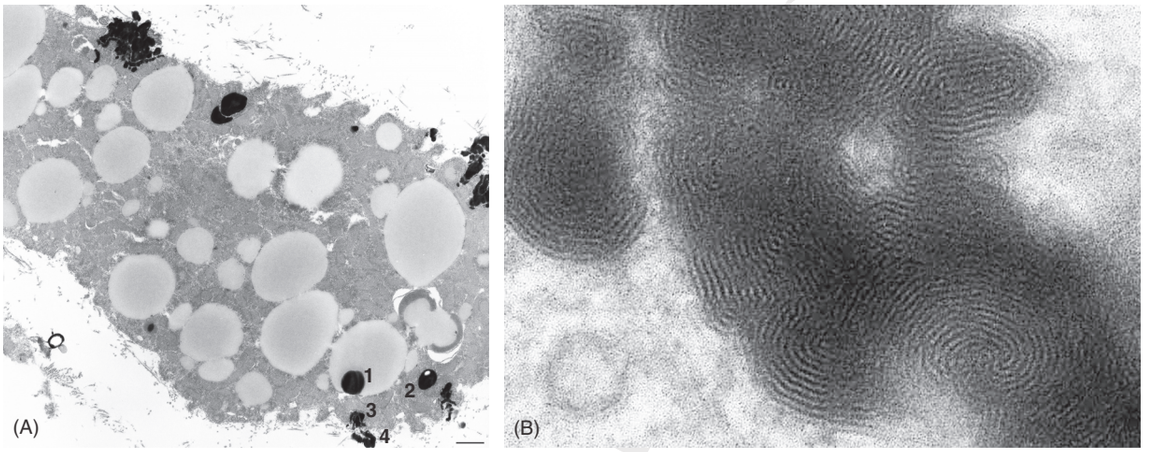

Figure 19 Morphologic aspects of lipolysis in slimming adipocytes. Electron microscopy (osmium-tannic acid cytochemistry). Bar: in A, 1.0 μm; and in B, 40 nm.

adipocytes (279, 468, 469) and the chronic positive energy balance can influence both neo-adipogenesis as well as expansion of mature adipocytes and the different reaction of subcutaneous versus visceral fat can produce relevant clinical differences (683).

It has been proposed that an altered mechanism of preadipocytes development (involving the BMPs system, see paragraph on origin of adipocytes) could be the cause for ectopic (visceral) fat accumulation with important clinical consequences (850).

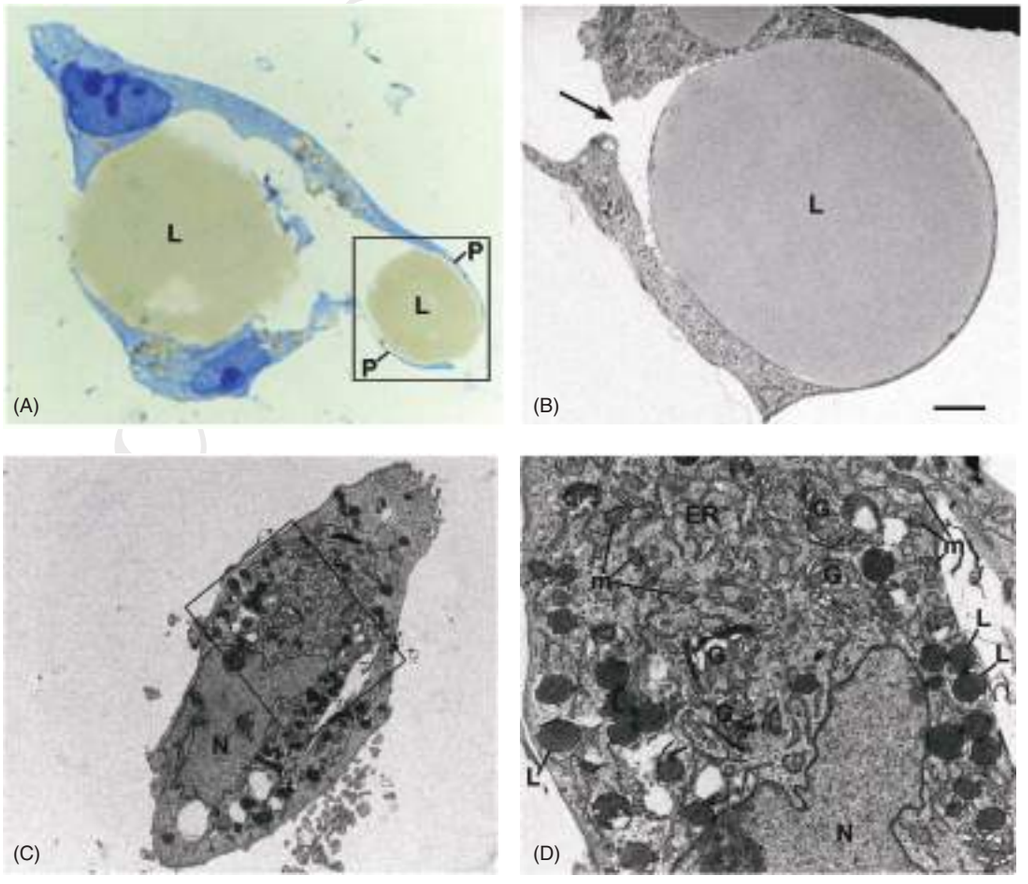

Figure 20 Critical steps of liposecretion process of mature adipocytes cultivated in primary culture (see also video: Fig. 21). Bar: in A, 2.5 μm; in B, 1.0 μm; in C, 1.5 μm; and in D, 0.5 μm. Adapted, with permission, from (576).

Figure 21 Still of time-lapse video reproducing a record of about 70 h of mature adipocytes in primary culture (see also Fig. 20; see video in 576). Adapted, with permission, from (576).

Thus, the cytological reaction of adipocytes to the positive energy balance as well as the site of reaction (subcutaneous or visceral) assume a clinical relevance mainly in relation to the adverse consequences of obesity.

In humans, the most important disorders related to an excess of adipose tissue accumulation (diabetes, cardiovascular diseases, hypertension) are correlated to the WHR ratio that is an index of visceral adipose tissue accumulation (central), suggesting an adverse potential of visceral adipose tissue in comparison with subcutaneous adipose tissue (67, 71). Importantly, it has been claimed that up to 85% of population variance in central abdominal fat is attributable to genetic influences (117).

The discovery that the TNFα is an adipose secreted factor with potential negative influence on insulin receptor physiology (407) and that can medate brown adipocytes apoptosis (637), caused a wide interest among scientists in search for a link between obesity and related disorders (mainly T2 diabetes). The well-known positive correlation between size of adipocytes and insulin resistance (870) opened the new perspective that expanded fat with enlarged adipocytes causes increased circulating levels of TNFα and consequent insulin resistance and subsequent T2 diabetes.

Chronic low-grade inflammation In 2003, two independent groups discovered that the obese WAT is infiltrated by macrophages creating a chronic low-grade inflammation (964, 979). Furthermore, they found a positive correlation between the size of adipocytes and number of macrophages infiltrating fat. It is well known that WAT can be artificially divided in two fractions by a method based on collagenase incubation and subsequent mild centrifugation. This method allows to separate the stroma-vascular fraction (SVF) from the floating fraction (FF) (72). FF contains the isolated mature adipocytes; SVF contains the rest of WAT including endothelial cells, adipocyte precursors, and inflammatory cells (171, 172).

Most of the cytokines produced by obese WAT (mainly: TNFα, IL-6, and i-NOS) were found in the SVF suggesting that macrophages and not hypertrophic adipocytes per se are the source of molecules with adverse effects on insulin receptor and that are able to cause, in long run, T2 diabetes (964, 979).

Death of adipocytes The cause of macrophages infiltration and establishment of a chronic low-grade inflammation is debated (93, 284, 444, 657, 795). We found that the overwhelming preponderance (>90%) of MAC2 immunoreactive (in active state of phagocytosis) macrophages infiltrating WAT of obese mice and humans are organized to form specific histopathologic figures denominated crown-like structures (CLS) (179) (Fig. 22). These structures are formed by CD206 immunoreactive (M2) macrophages surrounding an adipocyte-like structure. Frequently, giant multinucleated

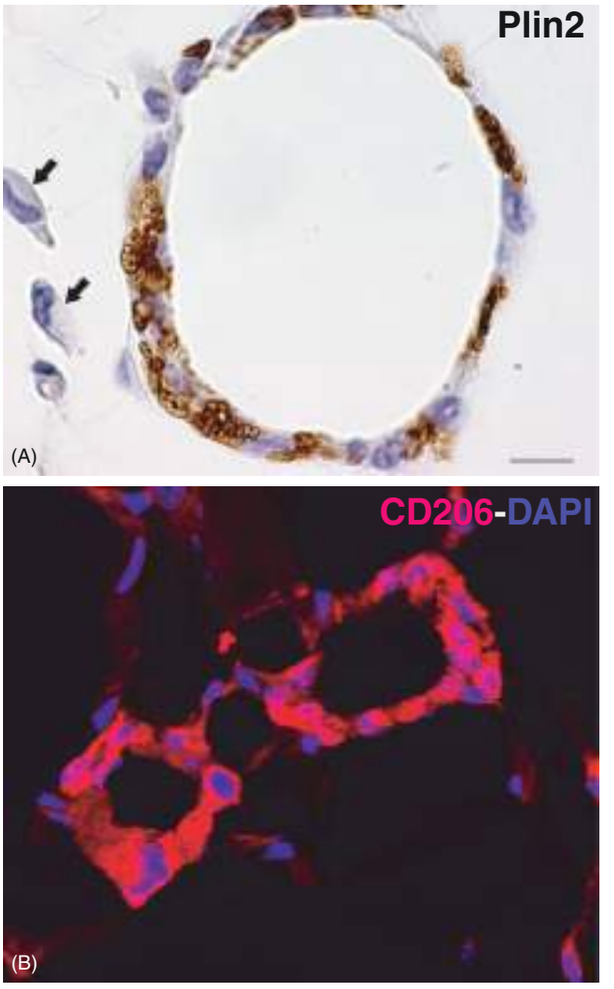

Figure 22 Immunohistochemistry of CLS showing M2 immunoreactivity of macrophages. Bar: in A and B 15 µm. A adapted, with permission, from (179).

MAC2 immunoreactive macrophages are also part of CLS. Immunohistochemistry and electron microscopy analyses revealed that CLS are sites of reabsorption of dead adipocytes. The largest part of adipocytes is formed by their lipid droplet, as a consequence most of the residual debris derived from adipocyte death is the lipid droplet, thus active macrophages reabsorbing the debris of death adipocytes (mainly the lipid droplet) form CLS in obese WAT. CLS are present also in lean WAT but their frequency resulted about thirty times higher in obese WAT (179) with a positive correlation between size of adipocytes and CLS density (612).

Subsequent elegant studies confirmed that adipocytes die both in lean and obese subjects and found that the average life of human adipocytes is about ten years (857).

To confirm that CLS are indeed due to death of adipocytes we used a well-characterized transgenic mouse model in which the death of adipocytes in adult mice is inducible and high synchronized. We used the "fat attac" transgenic model in which apoptosis is induced through forced dimerization of a caspase-8 fusion protein (613). The time course of histomorphological changes in epididymal and mesenteric WAT of these mice upon induction of caspase-8 dimerization showed that several adipocytes lost their immunoreactivity for perilipin1 (marker for alive adipocytes) and showed ultrastructural alterations of their organelles. The first population of inflammatory cells infiltrating the dying fat was made up by neutrophils, lymphocytes, and MAC-2 negative macrophages. In the following stages of the process, MAC-2 positive macrophages substituted for MAC-2 negative macrophages, followed by CLS formation. All perilipin1 negative dead adipocytes formed CLS (613). The reabsorption mechanism of macrophages in CLS has been recently described as exophagy (374).

Visceral adipocytes are more prone to death than subcutaneous adipocytes, as a matter of fact, in spite of a positive correlation between density of CLS and size of adipocytes, obese visceral fat with smaller adipocytes than those of subcutaneous fat show higher density of CLS suggesting that visceral adipocytes have a critical death size (size triggering death) lower than that of subcutaneous adipocytes (163,612). The cause of this different critical death size is unknown but several differences in secreted autocrine or paracrine factors by adipocytes or due to extracellular matrix components could play a role (683). Other explanations have been proposed: at least part of the visceral fat is whitened BAT, thus smaller than subcutaneous WAT and with different cellular proprieties (333). Very recently, a high propensity for death of whitened brown adipocytes has been shown (473). Interestingly obese db/db mice (lacking leptin receptor) with very large adipocytes (only hypertrophic fat, with visceral adipocytes size of about 11,000 μm^2) show about 3.5 times CLS density in visceral fat than obese ob/ob mice (lacking leptin), with hypertrophy and hyperplasia of fat (with visceral adipocytes size of about 8000 μm^2) (612). In line with these inflammation aspects, db/db mice develop T2 diabetes much earlier that ob/ob mice (513).

Bone marrow derived macrophages in peripheral tissues have been classified as inflammatory (M1) and anti-inflammatory (M2, mainly in phagocytosis state). A wide debate on the M1 or M2 state of macrophages infiltrating the obese fat is still open, but recent work indicates that the obese state recruit macrophages with a complex mixture of M1 and M2 phenotypes (380, 819). Moreover, some researchers did not observe an obesity-driven phenotypic switch of macrophages from an M2/anti-inflammatory to an M1/inflammatory state (981).

Among the effects of paracrine influences by the cytokines produced by macrophages, it has been proposed also a positive effect on adipogenesis that should play a role to reconstitute the adipocyte compartment of fat when inflammation produce massive death. In visceral fat of diet-induced obese mice, the number of dead adipocytes can be very high with about 70% of WAT occupied by CLS (873). This massive efferocytosis is followed by a prompt (few weeks) restoration of adipose population that imply a massive impulse to preadipocytes development. It has been shown that M2 macrophages of CLS secrete osteopontin (OPN) that is a potent chemoattractant for the subpopulation of CD44+ (receptor for OPN)/+ adipocyte precursors (505,508), suggesting a role for CLS macrophages in the recruitment of new adipose cells.

Interestingly a recent paper (563) support the idea that adipocyte precursors, under appropriate conditions, can shift toward fibroblast-like cells and be responsible for the characteristic fibrosis of human fat of obese patients.

Pyroptosis Fat inflammation and CLS density are positively correlated with adipocyte size (612, 964). To find the cause of death of adipocytes we studied a model of lean mice with hypertrophic adipocytes. Hormone sensitive lipase (HSL) is an important enzyme for lipolysis in adipocytes. Mice lacking this enzyme are lean but their adipocytes are hypertrophic. The density of CLS in fat of these HSL-knockout mice is similar to that found in WAT of genetically obese mice suggesting that size and not obesity per se, is responsible for adipocytes death (160). Interestingly, rosiglitazone administration to obese mice, reduced the size of adipocytes, the CLS density, and improved their glucose metabolism (482).

To try to understand the cause of death in hypertrophic adipocytes we studied the ultrastructure of subcutaneous and visceral fat of two murine models of genetic obesity (336). Several organelle alterations including irregular size and number of mitochondria, dilatation of rough endoplasmic reticulum, hypertrophy of Golgi complex was found in obese adipocytes that also showed glycogen clusters, calcium deposits, and increased amount of collagen fibrils associated with the external membrane. Most of these alterations (with the exception of calcium deposits) were quantitatively more abundant in visceral than in subcutaneous fat in booth strains. Furthermore, we also found, occasionally, cholesterol crystals in obese adipocytes. Notably, size and cholesterol content correlate positively in adipocytes (477). In line with

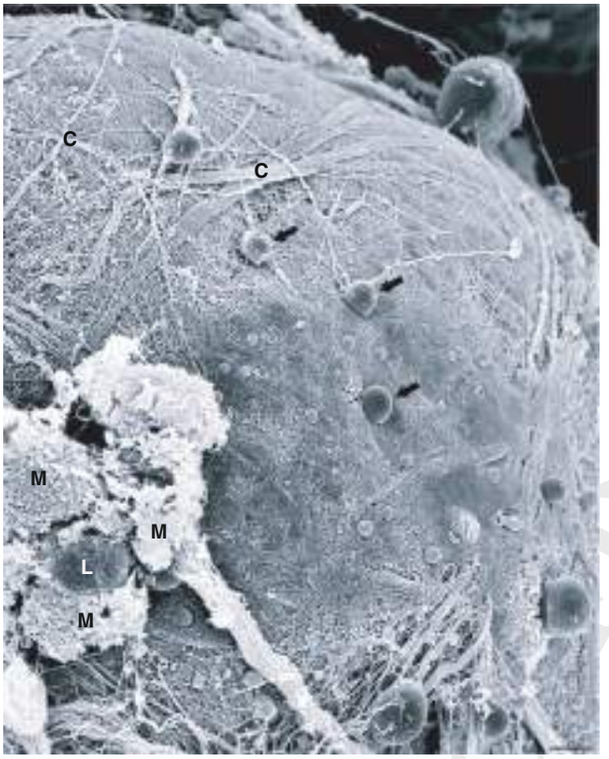

Figure 23 High-resolution scanning electron microscopy of a hypertrophic and degenerating adipocyte from an obese db/db mouse. Bar: 2 μm. Adapted, with permission, from (336).

these morphologic data suggesting a stressed phenotype of obese adipocytes, we found increased gene expression levels of some cell-stress markers: SOD-1, catalase, and GPX-1 (677).

We also found electron microscopy (both by transmission and scanning microscopy) pictures of degenerating adipocytes extruding lipid droplets with figures suggesting a prompt reaction by macrophages ready to surround and reabsorb the dying adipocyte (Fig. 23). The absence of perilipin1 in about 15% of obese adipocytes at immunohistochemistry corroborated the morphologic suggestion of degenerating adipocytes.

Hypertrophic and stressed adipocytes have hypertrophic Golgi complex and developed rough endoplasmic reticulum that are organelles implied in protein secretion, in line with the well-known hyper production of potent macrophage chemoattractant proteins such as MCP-1, CXCL14, MIP-1α, MCP-2, MCP-3, and RANTES by obese adipocytes (255,445,878,979). In human obese fat, the hyper production of collagen (365) and amyloid has also been shown (695).

The presence of cholesterol crystals and altered organelles are potential damage-associated-molecular patterns (DAMPS) known as activators of Nucleotide Oligomerization Domain receptors (NOD-like receptors, NLRs) that together with the precursor procaspase-1 and the adaptor ASC (PYCARD: Apoptosis-associated speck-like protein) form one of the protein complex of innate immunity known as inflammasome (255, 715, 741). The NLRP3 form of inflammasome has been shown to be activated in obese fat (940), and we observed that the gene expression levels of thioredoxin-interacting protein (TXNIP), which drives NLRP3 inflammasome activation following oxidative stress, were increased in obese fat. NLP3 activation results in production of activated caspase-1 that in turn processes the cytosolic precursors of the proinflammatory cytokines IL-1β and IL-18, which are secreted as biologically active cytokines that play important pathogenic roles for the development of insulin resistance (246, 425, 495, 661, 871). Caspase1 is a proinflammatory caspase whose activity can result in a highly inflammatory form of cell death known as pyroptosis (53, 566, 804).

We detected ASC, NLRP3 and caspase 1 in the cytoplasm of obese adipocytes (in genetically obese db/db and ob/ob as well as in HFD obese mice) by immunohistochemistry strongly supporting the idea that hypertrophic adipocytes die by pyroptosis (336). In line with this hypothesis the caspase-8 induced adipocyte specific death in the FAT ATTAC apoptosis model did not show any inflammasome related protein immunoreactivity in adipocytes (336).

In summary, considering all together, the sequence of events preceding the CLS formation could be: (i) hypertrophy of adipocytes due to positive energy balance, (ii) stress of hypertrophic adipocytes with organelles alteration, (iii) production of MCP1, with macrophages attraction to stressed adipocytes, (iv) degeneration and death of adipocytes by pyroptosis, and (v) CLS formation and reabsorption of debris derived from dead obese adipocytes. Of note, hypertrophic obese adipocytes are very big structures in comparison with the size of macrophages implying the need for multinucleated giant cell formation and a chronic phagocytosis activity. Several cytokines and factors produced by stressed adipocytes and macrophages in their different functional states are likely to be the molecular link between obesity and type2 diabetes (406, 546, 580, 666, 997). The lower critical death size of visceral fat offers an explanation to the well-known difference in metabolic-consequences between subcutaneous (pear shaped: protective) and visceral (apple shaped: inducing) obesity (73, 693, 929).

The concept of Critical Death Size Index As earlier detailed, two cell autonomous aspects are very important drivers of molecular mechanisms inducing the death of obese adipocytes: size and localization in the organ. As a matter of fact, in spite of a positive correlation between size and CLS density for both subcutaneous and visceral fat depots, visceral adipocytes die at lower size both in mice and in humans (103, 612). Thus, subcutaneous and visceral adipocytes display a different critical death size (CDS) (163). The CDS seems to be variable not only between subcutaneous and visceral fat but also in different areas of these depots, at different ages, different level of obesity and different genetic backgrounds, thus I propose a new parameter: critical death size index (CDSI) that take into account in the same time of the two variable aspects: size and death proneness indicated

by the CLS density in that specific area of adipose organ. The index results from the ratio between CLS density/area of adipocyte × 1000 and its main purpose is to give evidence to the unhealthy potential of the pathologic enlargement of adipocytes in that specific area of adipose organ. For example, in adult genetically obese db/db mice the inguinal (subcutaneous) adipocytes have an average size of about 14,000 μm^2 and a CLS density of about 250 CLS/10,000 adipocytes. Thus, the CLS index here is 250/14,000 × 1000 = 17. In the same mice, mesenteric adipocytes have an average size of about 11,500 μm^2 but the CLS density in this depot is about 1500 CLS/10,000 adipocytes, thus here the CLS index is 13 thus 7 times higher than that of inguinal fat. In the omentum of the same mice, the CLS index is 40, thus higher than inguinal but lower than mesenteric. Future applications of this index could be useful for a better understanding of the pathologic proneness of different fat depot in the adipose organ.

Adipose Organ Remodeling with Aging

Aging is a physiologic process, and several data link adipose organ remodeling with aging.

BAT quantity reduces with age. In a quantitative study of interscapular BAT of rats of different ages (from 1 week old to 104 weeks old) we showed a progressive decrease of multilocular adipocytes (*bona fide* brown adipocytes) in the interscapular area of adipose organ with a progressive simultaneous increase of unilocular adipocytes (*bona fide* white adipocytes). The percentage of multilocular versus unilocular in young animals was about 90% and progressively reduced to about 50% (793). Interestingly rats 104 weeks old exposed for 4 weeks at 4°C reversed the proportion approaching to that 1 week old, supporting the reversibility of the phenomenon (605).

In line with these old data, several recent papers showed a progressive BAT-WAT conversion also in other parts of the organ (499, 747). Interestingly loss and gain of function data support a role for orexin, a neuropeptide produced by neurons in the lateral hypothalamus (222, 779) in this aging-related remodeling phenomenon (810, 811).

Several data support similar reduction with age and BMI in humans (767, 946). In a case series of about 50 peri-carotid neck biopsies of adult patients we found UCP1 immunoreactive BAT in all patients below 30 years, in 20% to 30% of patients below 50 and in only 1 (60 years old) in 10 patients aged between 60 and 85. Furthermore, we found BAT in 90% of lean and only 10% of overweight patients; none of the obese subjects studied showed BAT (161, 1019).

This BAT reduction with age could be due to several age-related mechanisms including reduction of stem cells reproductive capacities, reduction of T3 production and T4-T3 conversion, reduction in mitochondrial function and of sympathetic nervous system activity (353, 746).

Interestingly PTEN (phosphatase tensin homolog, oncosuppressor gene) transgenic mice have enhanced BAT activity and live longer than controls (659). This is not surprising considering the antiobesity, antidiabetic, and antiatheroscerotic activity of BAT (26, 40, 625, 809, 865).

The concept of inflammaging imply a key role for chronic inflammatory states as aging factors and low grade of chronic inflammation of obese adipose organ (or meta-inflammation) is a paradigm of this concept (297). As a matter of fact, in meta-inflammation a dramatic increase in the number of cells expressing markers of cellular senescence [such as, p16, p53, and senescence-associated β-galactosidase (SAβ-gal)] in visceral adipose tissue (VAT) was found. Senescent cells (probably macrophages and T lymphocytes) secrete several factors, which are collectively known as a senescence-associated secretory phenotype (SASP) (298, 375, 833). These factors damage neighboring cells and contribute to age-related degeneration and inflammation that correspond to inflammaging.

Calorie restriction (CR) is a well-known antiaging mechanism across several species from yeast to primates (14, 65, 235, 295). At least part of the beneficial effects is thought to be due to the loss of adipose tissue that can be mimicked by surgical fat removal (80, 415). The molecular mechanism linking CR to lifespan is largely unknown but several data converge on the idea that adipose organ mediate cell-autonomous and cell-nonautonomous mechanisms linking miRNAs of fat and aging.

Dicer is an endoribonuclease with important effects on the microRNA pathway, which has a significant role in the differentiation and function of WAT and BAT (600, 602). It has been shown that adipose organ dicer reduces with aging and this reduction can be prevented by CR (601).

Dysregulation of miRNAs has been associated with several age-related diseases (245). Furthermore, miRNAs may influence pathways involved in senescence such as p53/p21 and p16/RB and pathways of proteins and transcription factors involved in senescence such as IL-6 and IL-8 and HMAG2 (61, 348).

The proposed cell autonomous mechanism should be related to miRNAs responsible for the renewal mechanisms of adipose tissue. Their alteration would induce insulin resistant hypertrophic adipocytes and inflammaging (601). The nonautonomous mechanisms would imply secretion of miRNAs influencing the rest of the organism (137).

Interestingly, fat specific dicer-KO accelerate aging and mitigates several effects of CR in mice (725).

Adipose Organ Remodeling in Humans

The anatomical organization of human adipose organ is very similar to that described above for murine adipose organ (304): that is, a large and diffuse organ composed by a mixture of WAT and BAT, weighting about 20% of the total body weight (adult lean) and mainly distributed in two compartments of the body: subcutaneous and visceral, but with extensions into several other organs such as muscles, heart, bone marrow, parotid, parathyroid, gastrointestinal apparatus,

lymph nodes, joints, and visual apparatus. The most relevant differences between humans and rodents reside in the relative amount of BAT, its anatomical localization and in the organization of subcutaneous WAT.

Several aspects of human adipose organ have been specifically described in previous paragraphs of this review and only a general view of its anatomy and remodeling is reported here.

BAT

Immunohistochemistry studies and clinical imaging techniques (mainly based on positron emission tomography: PET) have been used quite recently to confirm old data (498) showing the presence of metabolically active BAT in adult humans (211, 608, 626, 776, 938, 948).

Human BAT is composed similarly to murine BAT (Fig. 2): that is, UCP1 immunoreactive multilocular adipocytes organized in polygonal lobules densely innervated by TH immunoreactive (noradrenergic) parenchymal nerve fibers and provided with a dense vascular supply (Fig. 24).

In the adipose organ of adult humans, it is rare to find large areas composes exclusively by BAT. Even the more compact areas are in fact "infiltrated" by unilocular adipocytes, thus resembling the mixed depots of murine adipose organ (1019). Electron microscopy show the typical numerous large and packed with laminar cristae mitochondria. Several adipocyte precursors have been described in pericarotid BAT of human adults. Quantification of these brown adipocyte precursors identified by their specific ultrastructural characteristics (see development paragraph) suggest a frequency of these cell of about 1/5-10 pericapillary areas (1019). Their ultrastructure was very similar to that found in brown adipocyte precursors of hibernomas, where their frequency was about five times higher (561).

In adult female Sv129 mice (obesity-resistant), maintained for ten days at 28°C, quantitative analyses showed that about 60% of the total number of adipocytes contained in the adipose organ were brown adipocytes. Instead, in the obesity-prone mice B6 (same experimental conditions as Sv129) only about 15% of all adipocytes were brown adipocytes (614,950), supporting a role for the genetic background for obesity prevention.

Figure 24 TH immunoreactivity of parenchymal noradrenergic fibers in human brown adipose tissue. Bar: 23 μm.

Precise anatomical quantification studies of human BAT does not exist, but, in adult lean humans maintained in a standard western-type environment, BAT have been calculated in about 100-150 gr (771,937), thus representing a very low percentage (about 1%) of the human adipose organ weight, but even a prudent estimate of 50 g of active BAT in adult humans would mean a potential impact on BMR of 2.7% to 5% that outline its potential clinical relevance (210). Furthermore, it must be outlined that BAT activity is highly dependent from age, sex, BMI, and environmental conditions living open the door to the possibility to increase the amount of active BAT in adult humans (917). Like in small mammals, obesity and aging inversely correlate with the presence of BAT and in old rats, we found an about 50% reduction of interscapular BAT that can be entirely reconstituted by cold exposure (605, 793). As a matter of fact, it has been clearly shown also in humans that the amount of active BAT can be increased by acute and chronic cold exposure and by administration of the last generation β3AR agonist mirabegron and capsinoids (212, 775, 938).

The therapeutic impact of a chronic activation of BAT in adult humans to treat obesity and related metabolic disorders is still unknown, but several data suggest a positive impact on insulin sensitivity of cold exposure (144) in line with previous studies showing a reduced expression of brown adipogenic genes in subcutaneous fat of adult humans with insulin resistance (994).

Furthermore, it must be outlined that under appropriate conditions not only quiescent BAT become active, but also WAT of adult humans can convert to BAT.

Omental fat is a classic WAT of adult humans and the analyses of biopsies from 20 lean adult patients confirmed the presence of only unilocular UCP1 negative adipocytes in this tissue. Omental biopsies from 12 patients suffering from pheochromocytoma (benign tumor of adrenal glands secreting adrenaline and noradrenaline) showed a reduction in size of unilocular adipocytes in all pheochromocytoma patients and browning in half patients (307). Browning was accompanied by a classic remodeling of the tissue with increased density of capillary network and of noradrenergic parenchymal nerve fibers. Interestingly in all patients showing browning we found not only UCP1 immunoreactive multilocular adipocytes but also UCP1+ paucilocular adipocytes (UCP1-PL), probably representing the first step of white-brown conversion (see browning paragraph). The electron microscopy of UCP1-PL revealed the presence of abundant mitochondria with intermediate features between those of white-like and brown-like mitochondria and rare intermediate forms, that is, mitochondria with an area with brown-like characteristic (roundish with laminar cristae) and an area with

white-like characteristic (elongated with randomly oriented cristae). Interestingly, very similar intermediate mitochondria were found in transforming human brown adipocytes maintained in primary culture (152). The master transcription factor of browning PRDM16 was detected by immunohistochemistry only in nuclei of cells with morphologic features of converting adipocytes (i.e., mainly in paucilocular adipocytes and multilocular adipocytes) (307). Some multilocular adipocytes resulted PRDM16 negative suggesting a role for the acquirement of the brown phenotype but not for its maintenance.

The origin of brown adipocytes found in the omental fat of pheochromocytoma patients seems, therefore, to be due to direct conversion of preexisting white adipocytes and data obtained with the proliferation marker Ki67 and quantitative electron microscopy of preadipocytes fully confirmed this idea. Ki67 was negative in multilocular cells (with internal positive cells in a lymph-node). Preadipocytes show specific features that can be visualized by electron microscopy, and a quantitative evaluation showed no difference with omentum samples of normal subjects and allowed us also to exclude the possibility that preexisting preadipocytes developed without proliferation because intermediate forms between preadipocytes and mature multilocular cells were not detected.

Thus, the main phenomenon giving rise to brown adipocytes in omental fat of adult humans in these conditions seems to be a white to brown direct transdifferentiation of mature adipocytes. The fact that visceral human white adipocytes of a depot (omentum), that is usually resistant to browning even in small mammals (169), can convert to brown adipocytes offer a new important therapeutic target (333).

PET studies showed that the activated BAT in humans is distributed in the areas surrounding aorta and its main branches (483), thus in the visceral part of the adipose organ including neck (perisubclavian and pericarotid fat), thorax (periaortic and periintercostal fat), and abdomen (mainly: periaortic and perirenal). It must be outlined that the supraclavicular region is one of the sites of branches of aorta (subclavian arteries) and this site is considered the main or classic anatomical site for BAT in adult humans, but other periaortic regions (such as perirenal fat) have been proven as sites of BAT not only in adults but also in old patients (161, 169).

Thus, the main depot for human BAT is visceral and close to the aorta system. This anatomical location offers an easy finalistic reason: the aorta system is ready to distribute heat produced by BAT to the rest of the body. In small mammals (mice, rats, and ferrets), the main depot for BAT is located in the interscapular and surrounding regions (subscapular and deep cervical). These areas are located in part in the subcutaneous depot (interscapular) and in part in deep intermuscular regions (subscapular and deep cervical) in direct anatomical continuity with the anterior subcutaneous depot and the vascular system is connected with these BAT-rich areas with special vessels that do not exist in humans such as the Sulzer's vein (624). The reason for this difference could depend on the fact that small mammals need more thermogenesis than humans' due to differences in heat dispersion caused by a different body volume/surface ratio that favors heat dispersion in small mammals (396). This difference requires extra BAT for small mammals that is located outside the trunk, but still in tight connection with the central vascular system through the Sulzer's vein connecting interscapular BAT with azygos vein. This location of BAT mainly in the subcutaneous compartment together with the widely accepted concept of subcutaneous inguinal fat proneness to browning probably contributed to the idea that subcutaneous fat should be the main target for browning. It should be outlined that some parts of this big depot are located deeply among skeletal muscles (subscapular and deep cervical) and therefore they are not strictly located in the subcutaneous compartment (between skin and skeletal muscle fascial plane). Furthermore, most data from murine adipose tissues studies come from epididymal fat used as a paradigm of visceral fat and its resistance to browning contributed to the aforementioned idea of subcutaneous target, but it must be outlined that epididymal fat is not in direct connection with the aorta system and cannot be taken in consideration as representative of the visceral compartment (169).

Thus, if the visceral fat should be the target for browning, the earlier reported data on browning of human omentum are particularly remarkable taking also into consideration the importance of visceral fat for the metabolic consequences deriving from its hypertrophy in obesity (73, 238, 929).

WAT

The vast majority of fat in human adipose organ is WAT (Fig. 25). Subcutaneous WAT is located in the compartment located below skin. Skin is composed by epidermis and dermis. Murine dermis contains WAT (175), human dermis contains WAT only in proximity to adnexa such as bulbs of hairs and sweat glands (169, 251). This WAT is continuous with subcutaneous fat that form a layer below skin of variable thickness and can be subdivided in two parts: superficial and deep (108). The visceral fat is distributed in the trunk similarly to that of murine adipose organ. Mediastinal fat is continuous with that surrounding the aorta and its main branches till the deep areas of the neck and axillae. Interestingly epicardial fat exhibits intermediate features between white and brown adipocytes (773). In the abdomen, it is located in intraperitoneal compartments (mainly: mesenteric and omental fat) or below the parietal peritoneum (mainly: perirenal and pelvic fat) (68, 411).

Distribution and thus, in a broad sense remodeling, because always associated with functional variations of adipocytes (16, 19, 21, 237, 299), of fat in subcutaneous or visceral compartments is mainly regulated by sexual hormones. The gender differences in human adipose organ distribution are more evident than in murine adipose organ (69, 70, 780, 894, 895). Females accumulate fat mainly in the

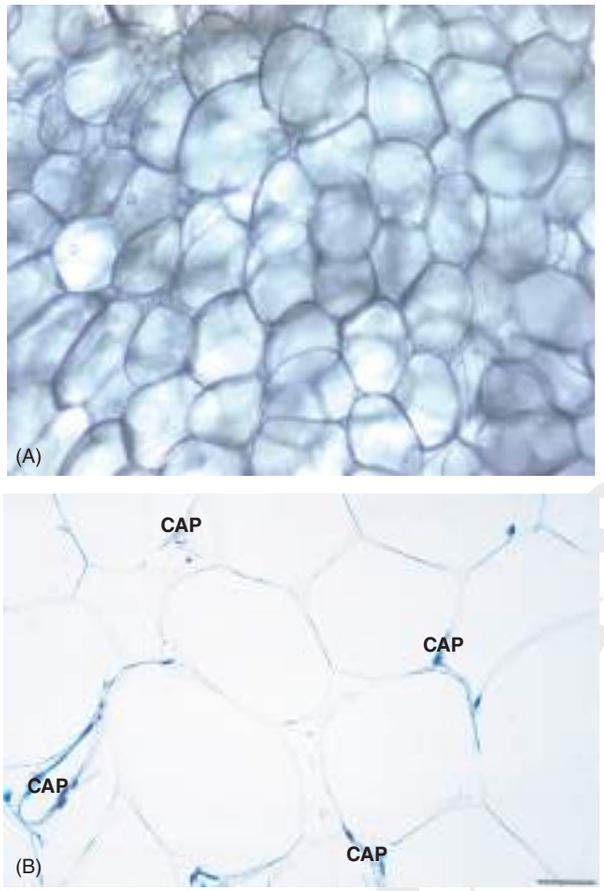

Figure 25 Human subcutaneous white adipose tissue fresh (A) and paraffin embedded (B). Bar: in A, 80 μm; and in B, 30 μm. B adapted, with permission, from (153).

subcutaneous compartment with preference of areas corresponding to the mammary glands localization in other mammals, that is, in the breast and in the gluteo-femoral areas. Males have more visceral fat than females: it has been calculated in lean adult subjects adjusted for their total body fat, females have about 60% of the visceral fat found in men (197, 223, 231, 514). The differences disappear after menopause (912, 1004) and studies on subjects under hormonal treatment for female-to-male and male-to-female transsexuals confirmed the importance of sexual hormones in fat distribution (259-261).

These differences are mediated also by different activity of LPL, that is, the enzyme favoring the lipid accumulation in adipocytes by uptake from circulating fatty acids and triglycerides (18, 719). LPL activity is higher in visceral fat of men and in subcutaneous fat of women. Furthermore, testosterone inhibit LPL activity in subcutaneous fat of men. Another mechanism explaining the sexual differences imply the different distribution of adrenergic receptors (AR): estradiol increases the antilipolytic α2AR in subcutaneous fat (681). The ratio of α2AR to βARs in subcutaneous fat in premenopausal women is high and reversed in visceral fat favoring lipolysis. In men and postmenopausal women, the ratio is reversed offering an explanation for preferred visceral fat accumulation (322, 733, 902). Also, the distribution of the two estrogen receptors (ERα and β) contribute to the different fat localization (615, 670). Mice lacking ERα have an increased body fat with preferential increase in the visceral compartment (217, 390), in line with several data suggesting a lipolytic effect of ERα and opposite effect of ERβ and with the relative lack of expression of ERα in visceral fat of males (243, 671).

Furthermore, the expression of ERα in central nervous system could play a role in fat distribution. ERα seems to be more expressed in hypothalamic neurons responsible for the adrenergic innervation of visceral fat and a target disruption of ERα in the ventromedial hypothalamus induces visceral obesity in female mice (3, 183, 982).

In line with these data experiments on denervated fat pads and central administration of estrogen demonstrated important peripheral effects due to central nervous system stimuli of ERα. Mainly based on these data, it has been proposed a theory explaining the different gender distribution of fat. It is based on three main drivers: sympathetic nervous system (SNS) activity, adrenoceptors, and LPL activity. The increased SNS activity on visceral fat due to the hypothalamic activation by estrogens would result in less visceral fat in women. In addition, the higher leptin production by subcutaneous fat would reinforce the same SNS activity. These stimuli and the aforementioned different distribution of antilipolitic (α2) and lipolytic (β1-3) adrenoceptors together with the different LPL activity of adipocytes would explain the different sexual distribution of adipose organ.

Teleological explanations for gender differences in fat distribution have been proposed: the visceral fat would allow a readier disposal for short-term energy requirements typical for men hunting and combatting. The lower lipolytic rates in subcutaneous fat make this depot more appropriate to respond to chronic metabolic challenges such as those due to pregnancy and lactation periods (671).

In any cases, this sexual dimorphism has important healthy consequences when fat accumulation reaches the overweight or obesity state: only visceral fat accumulation has severe metabolic consequences (see also obese organ paragraph) (236, 480, 701).

The human fat of obese patients is inflamed with all the histology characteristics described earlier for murine obese fat, including the CLS (179).

In patients with unusually large adipocytes, we found structures similar to gigantic CLS that we described as cyst-like-structures (CyLS) (103). In this study, we showed that the size of adipocytes was larger in subcutaneous than in visceral fat in all patients, but the number of CLS was higher in visceral than in subcutaneous fat suggesting that, similarly to murine fat, also in humans the critical death size is smaller in visceral than in subcutaneous fat and offering an explanation to the old notion that visceral fat accumulation is more

dangerous for metabolic consequences than subcutaneous fat accumulation. Diabetic patients showed the highest level of CLS density in line with the idea that inflammation is linked to metabolic consequences (103).

After bariatric surgery and substantial weight loss subcutaneous fat histology showed a reduction in adipocyte size and in CLS density in line with previous work (106), but a diffuse infiltration of macrophages was still present in most diabetic patients in association with a persistence of altered pancreatic β-cell glucose sensitivity.

Adipose Organ Remodeling in Lypodystrophic Mice and Humans

Congenital and acquired lipodystrophies are rare diseases (about 1:1,000,000) affecting mainly the adipose organ (319, 520). It can affect all parts of the adipose organ (generalized), limited to specific areas (partial), or localized (trivial loss of fat). Genetic causes have been identified for *congenital generalized lipodystrophies* in humans including mutations in the following genes:

1. AGPAT2, encoding an acyltransferase resident in endoplasmic reticulum of adipocytes and responsible for de novo phospholipid and triglycerides biosynthesis, (4, 319, 320).

2. BSCL2, encoding seipin, a protein resident in endoplasmic reticulum of adipocytes and involved in the normal formation of lipid droplets, (558, 936).

3. CAV1, encoding caveolin1, a main component of caveolae plasma membrane in several cell types. Adipocyte membranes are rich in caveolae, which increase ten times during differentiation *in vitro* (274) and that are involved in the lipid droplet development (457, 663).

Moreover, familial partial lipodystrophies:

1. LMNA, encoding for two major proteins: prelamin A and lamin C plus lamin AΔ10 and C2 by alternative splicing. Posttranslational modifications of prelamin A induces the formation of mature lamin A. Lamins are members of intermediate structural nuclear filaments of inner surface of nuclear lamina that are efficiently processed to allow several basic functions of the cells permitted by fine chromatin conformational changes, affecting DNA damage response factors and shuttling transcription factors (104, 116, 362, 607).

2. ZMPSTE24, encoding a prelamin-proteolytic processing zinc metalloproteinase necessary for lamin A formation (842).

3. PPARG, encoding PPARγ a key transcription factor in adipogenesis (759).

4. AXT2, encoding protein kinase B, a phosphoinositide-dependent serine/threonine kinase involved in postreceptor insulin signaling (325).

Anyway, the most prevalent form of lipodystrophy is an acquired form that occurs in HIV-infected patients chronically treated with protease inhibitor therapy (418).

Several of the generalized forms (both congenital or acquired) have a common trait: fat misdistribution with absence in subcutaneous areas mainly in the lower part of the body and abundance of fat in the visceral compartments of the upper part of the body (268, 659, 799).

This upper-body location corresponds, at least in part, to the usual anatomic distribution of human BAT (see the human adipose organ paragraph) and several data suggest that this fat could indeed be a modified BAT both in murine models and in human patients (see the succeeding text).

Most of the patients suffering for lipodystrophy present symptoms similar to those of obese patients such as insulin resistance, hyperglycemia, hepatic steatosis, hypertension, and dyslipidemia (360, 413, 769, 788, 789). This paradox clearly suggests that either pathologic excess or reduction of adipose organ induce similar metabolic alteration and a balanced energy storage and utilization are necessary for normal homeostatic mechanisms (943) (Fig. 26).

Several murine models of lipodystrophy (787) have been created or described including:

1. Mice with adipocyte-specific expression of diphtheria toxin (766).

2. A-ZIP/F mice, which were rendered lipoatrophic by artificial interference with the adipogenic C/EBP transcription factors (527, 594). About 99% of WAT loss (727).

3. FAT-ATTAC mice, which undergo transient lipoatrophy after temporal induction of adipocyte apoptosis (613, 669).

4. aP2-nSrebp1c mice, which express a truncated, constitutively active Srebp-1c transgene in adipocytes, and whose lipodystrophy phenotype is poorly understood (830). About 70% of WAT loss with abnormal BAT hypertrophy (829).

5. fld ("fatty liver dystrophy") mice, which suffer from severe lipoatrophy because of spontaneous mutations in the Lpin1 gene that is a transcription activator necessary for adipocyte differentiation (upstream of PPARγ) (685, 688, 730).

6. Mice deficient for Lmna (gene encoding nuclear lamins) (see the preceding text) (208, 258).

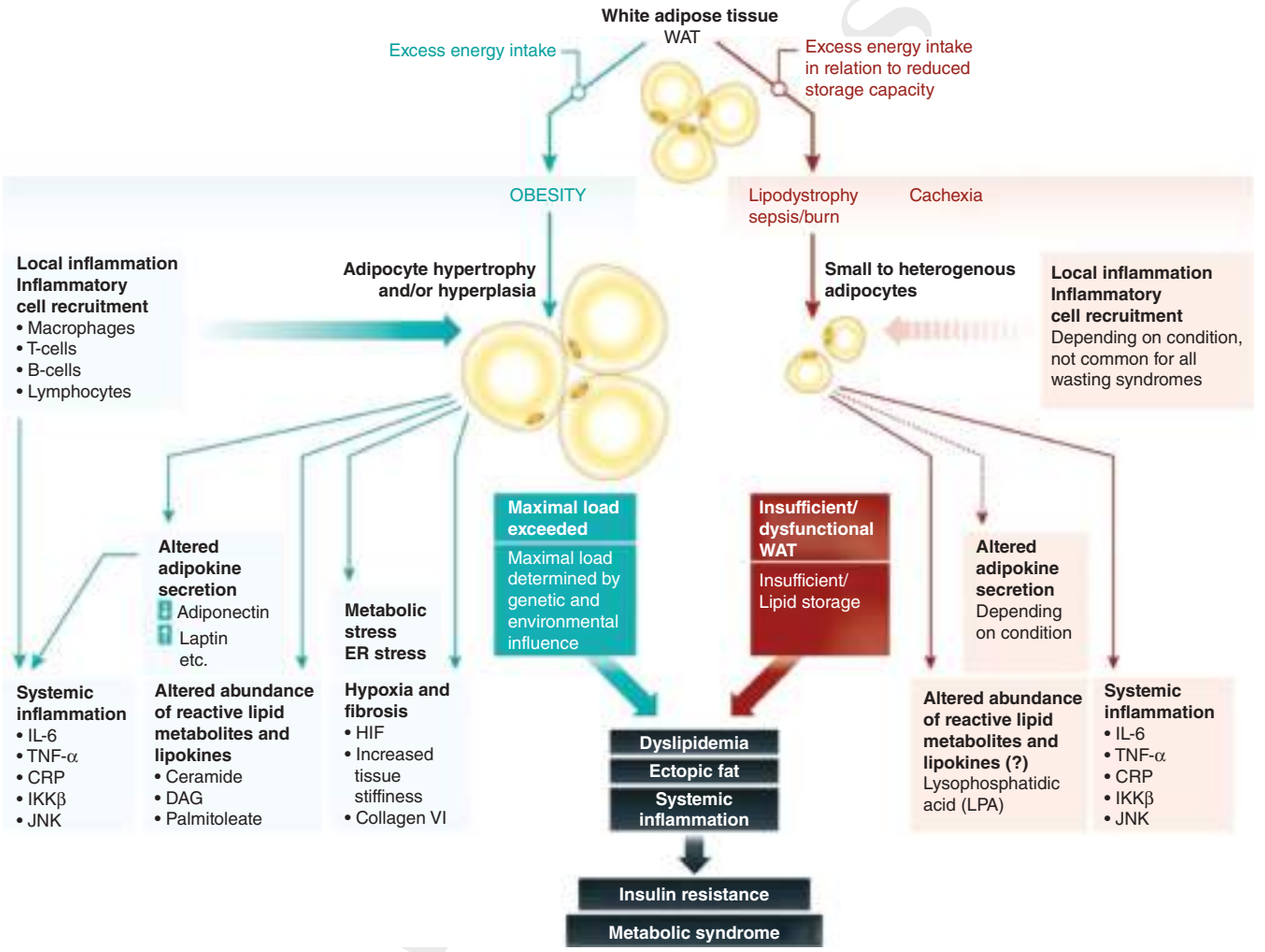

Figure 26 Proposed mechanisms for the paradoxical clinical outcome of obesity and lipodystrophy. Adapted, with permission, from (943).

7. Zmpste24 is a gene encoding for a metallopeptidase involved in the processing of Lamin A (684).

8. Ribosomal S6 kinase (Rsk2). RSK2 is a member of a family of growth factor-regulated kinases abundantly expressed in WAT and probably involved in a variety of physiologic processes. Its functional role is still elusive. Knockout mice showed about 50% of WAT loss (258).

9. Insulin receptor ablation in 80% and 98% of the cells resulted in marked and diffuse lipoatrophy due to lack of differentiation of adipocyte precursors (175, 464). Interestingly, in localized lipodystrophy due to insulin injections in subcutaneous fat in patients with T1 diabetes, we found numerous poorly differentiated adipocytes and slimmed cells (588). We proposed that a localized insulin resistance caused by insulin injections could be responsible for both: lack of development on preadipocytes and prevalent lipolysis in differentiated adipocytes (Fig. 27)

10. Irs1/Irs3 double-knockout mice show diffuse lipoatrophy (497).

11. Adipocyte-specific Pparγ-null mice show diffuse lipoatrophy with hypertrophy of abnormal brown adipocytes (388, 422, 434).

12. PTEN-Myf5-Kcnoc PTEN (Phosphatase and Tensin homolog) is a tumor suppressor that counteract the activity of phosphatidylinositol 3-kinase type I (PI3K). Myf5 is expressed by precursors of brown adipocytes in interscapular and perirenal BAT (808). Myf-5 driven ablation of PTEN give rise to a partial lipodystrophy with atrophy of WAT and hypertrophy of abnormal BAT (659, 783).

13. $Pparg^{ldi}$. This partial lipodystrophy was the serendipitous outcome of a genetically engineered allele of peroxisome proliferator-activated receptor (PPARγ); the phenotype was atrophy of WAT and hypertrophy of abnormal BAT (460).

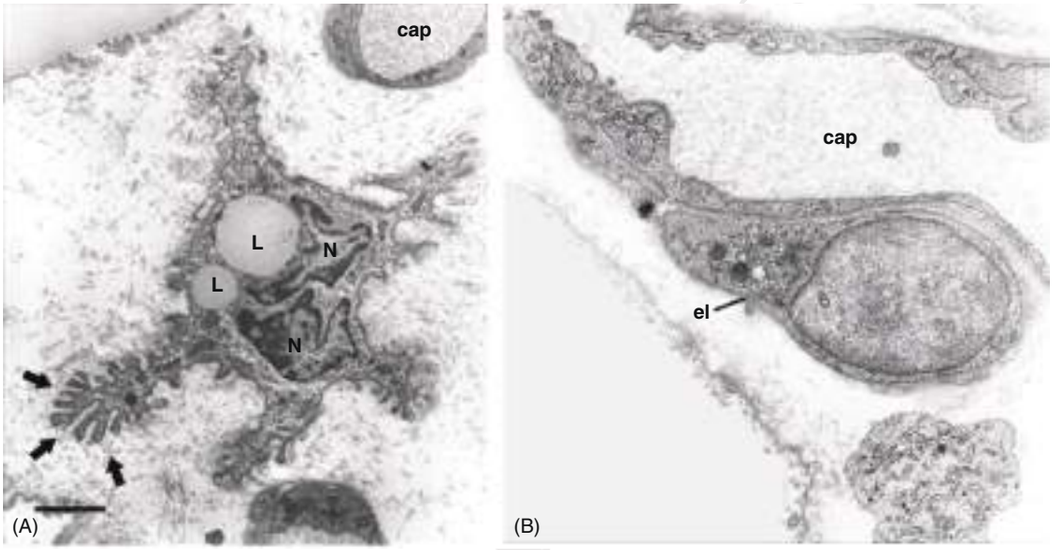

Figure 27 Slimmed adipocyte (A) and adipocyte precursors (B) resulted typical features of localized lipodystrophy secondary to insulin injections. Bar: in A, 1.8 μm; and in B, 1.2 μm. Adapted, with permission, from (588).

Interestingly, several murine models of lipodystrophy (aforementioned in 4, 6, 7, 11, 12, and 13) showed a misdistribution and remodeling of the adipocytes in the adipose organ with variable atrophy of subcutaneous WAT and hypertrophy of abnormal BAT. The abnormal BAT was mainly characterized by hypertrophic adipocytes with a unilocular-like morphology (i.e., BAT-whitening).

Human lipodystrophy with similar characteristics are:

1. Multiple symmetric lipomatosis (MLS) of type 1 (Madelung disease). Rare acquired disease in adult red wine drinkers with symmetrical accumulation of fat masses in all typical sites for BAT areas and atrophy of WAT in the rest of adipose organ (268). Histology and electron microscopy of biopsies from lipomatosis areas showed unequivocal signs of morphological markers of modified BAT both in mature adipocytes of the tissue and in primary cultures of developing adipocyte precursors from the stroma-vascular fraction extracted from the MLS tissue (176, 639, 1005). Interestingly, the red wine is rich in resveratrol that is a potential stimulator of BAT (489). Recently a mutation in gene coding for hormone sensitive lipase in a case of multiple symmetric lipomatosis has been reported (1020).
2. Familial partial lipodistrophy (FPLD). The most common subtype is FLPD2 (about 500 reported patients) (198, 413). Due to a missense mutation of LMNA, it is an autosomal dominant disorder characterized by subcutaneous fat loss, with pubertal onset, from the upper and lower extremities and often fat accumulation in the face, neck, perineal, and intra-abdominal areas, particularly in women.
3. Highly active antiretroviral therapy–induced lipodystrophy (HAART) in patients infected with human immunodeficiency virus (HIV). This is the most frequent acquired lipodystrophy (128, 289, 645, 923). After 2 to 4 years of HIV-1 protease inhibitors (PIs) or nucleoside reverse transcriptase inhibitors (NRTI) therapy a partial lipodystrophy occurs with loss of subcutaneous fat from the extremities and face and fat accumulation in the BAT areas.

PIs inhibit ZMPSTE24 that is an important enzyme in the process of prelamin A to lamin A, thus inducing accumulation of toxic prelamin A (similar to FPLD2, see the preceding text). NRTI cause mitochondrial dysfunction due to inhibition of mitochondrial polymerase γ. A brown lineage signature has been shown in biopsies from lipodystrophic fat of these patients too (128, 910).

Thus, several murine models of lipodystrophy and the most frequent human congenital and acquired lipodystrophies have the WAT/BAT common trait described earlier.

One very simply explanation come from the general concept of adipose organ remodeling described in this review: mammals have an organ composed by two different tissues: WAT and BAT. Usually different tissues in the same organ cooperate to a single finalistic role useful for the organism homeostasis. Apparently, WAT (energy storing) and BAT (energy dissipation) have quite distinct and even opposite functions, but they cooperate in special occasions by the reversible transdifferentiation property: when chronic cold requires intense thermogenesis WAT browning increase the thermogenic BAT. When

chronic positive energy balance requires more storing tissue, BAT whitening conversion help to reach the goal. Thus, it could be hypothesized that lipoatrophy of WAT induce a transformation of the remaining part of the adipose organ (BAT) that adapt the cells from a thermogenic to a storing cell type. This transformation seems to be incomplete and residual markers of the original BAT tissue can be detected (128, 176, 639, 910, 1005). Of course, other mechanisms could concur to develop the abnormal hypertrophic BAT.

Conclusions

This organ was largely unexplored until about 50 years ago when the obesity became a diffuse clinical condition in the western countries and the first connections with metabolic syndrome where realized. Since then a progressive interest for adipose tissues was demonstrated by an exponential increase in the related scientific literature. Nowadays, the metabolic syndrome represents one of the most important worldwide healthy problem and the key role of adipose organ is widely recognized. Several matters are still largely debated such as the origin of adipocytes and their plastic adaptability to the thermogenic, metabolic and lactating needs of the organism, but several data appear widely accepted such as those related to the concept of meta-inflammation. The lack of specific medical treatment is one of the cause that push the scientific community to tackle the myriad of molecular mechanisms still unexplored in the physiology and physiopathology of this important and complex organ and I sincerely hope that this review could play a role in stimulating new scientists to join the cohort that dedicated their scientific life to study this fascinating organ.

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