Harokopio University

School of Health Science and Education

Department of Nutrition and Dietetics

Graduate Program – Master's Degree

Nutrition and Exercise

BROWNING AND WHITENING OF SUBCUTANEOUS WHITE ADIPOSE TISSUE AFTER SEVERE BURN INJURY

Master Thesis

of

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1. Greek Abstract (Περίληψη)

Εισαγωγή: Ο καφέ λιπώδης ιστός προσέλκυσε το επιστημονικό ενδιαφέρον αφού διαπιστώθηκε η παρουσία του στους ενήλικες ανθρώπους. Αργότερα ανακαλύφθηκε ένα ακόμα είδος λιποκυττάρων, τα οποία εμφανίζουν παρόμοια χαρακτηριστικά με τον καφέ λιπώδη ιστό και ονομάστηκαν μπεζ λιποκύτταρα. Στη συνέχεια έρευνες διαπίστωσαν ότι ο λευκός, καφέ και μπεζ λιπώδης ιστός έχουν την δυνατότητα να εναλάσσονται μεταξύ τους, υπό την επίδραση συγκεκριμένων ερεθισμάτων.

Σκοπός: Υποθέσαμε ότι η ενεργοποίηση του καφέ λιπώδους ιστού σε ασθενείς με εγκαύματα είναι αναστρεφόμενη και σκοπός ήταν η διευκρίνηση της χρονικής στιγμής κατά την οποία πραγματοποιείται η αντίστροφη διαδικασία.

Μέθοδος: Οι συμμετέχοντες στην έρευνα ήταν παιδιά με εγκαύματα που κάλυπταν ≥30% της επιφάνειας σώματος. Μέρος των ασθενών μελετήθηκαν προοπτικά κατά τη διάρκεια παραμονής στο νοσοκομείο και μετά το εξιτήριο μέχρι και 1 χρόνο μετά το έγκαυμα. Ο Βασικός Μεταβολικός Ρυθμός (BMP) μετρήθηκε με έμμεση θερμιδομετρία, ο υπολογισμός της γονιδιακής έκφρασης με real-time PCR και η μιτοχονδριακή δραστηριότητα με σπιρομέτρηση υψηλής ανάλυσης.

Αποτελέσματα: Συνολικά, 224 παιδιά ηλικίας 11±5 χρονών (31% θυληκά, 65% αρσενικά) με σοβαρά εγκαύματα που κάλυπταν το 55±17% της επιφάνειας σώματος μελετήθηκαν. Η γονιδιακή ανάλυση έδειξε ότι η έκφραση των CIDEA PRDM16, TBX1, UCP1, UCP2 και UCP4 αυξήθηκε σταδιακά, των CITED, FGF21 και UCP3 μειώθηκε, και των ZIC1 και TMEM26 κυμαινόταν. Τα επίπεδα του BMP αυξήθηκαν σημαντικά αμέσως μετά το έγκαυμα (1-45 μέρες) και στη συνέχεια έφτασαν σχεδόν τα υγιή επίπεδα 1 χρόνο μετά το έγκαυμα. Η μιτοχονδριακή δραστηριότητα αυξήθηκε φθάνοντας τις μέγιστες τιμές της 1 μήνα μετά το έγκαυμα και στη συνέχεια μειώθηκε σταδιακά επιστρέφοντας στα επίπεδα της πρώτη εβδομάδας.

Συμπεράσματα: Η ενεργοποίηση του καφέ λιπώδους ιστού από την επίδραση του αδρενεργικού στρες που αναπτύσεται μετά από έγκαυμα αντιστρέφεται φυσιολογικά όταν το ερέθισμα (αδρενεργικό στρες) εξαφανίζεται. Η κατανόηση των υποκείμενων μηχανισμών μπορεί να έχει απώτερη εφαρμογή στην κλινική πράξη, για παράδειγμα στην θεραπεία παχύσαρκων και εγκαυματιών ασθενών.

Λέξεις-κλειδιά: καφέ λιπώδης ιστός, πλαστικότητα, αδρενεργικό, μιτοχόνδρια, φαιοποίηση

2. English Abstract

Introduction: Brown adipose tissue attracted the scientific interest after it was confirmed that it is present in human adults. Later, another type of brown-like adipocyte was found, called beige. Research in the field revealed the occurrence of two transdifferentiation processes under certain stimulation, named browning and whitening.

Hypothesis: The hypothesis was that whitening process occurs in burned patients following the browning process. Also, we aimed to investigate how much time post-burn, the whitening process happens.

Methods: For this study, children with ≥30% TBSA were included. A subset of burn patients were prospectively followed during their hospitalization and 1 year after discharge. Browning and whitening of sWAT was studied by measuring REE with indirect calorimetry, quantifying gene expression of brown markers with real-time PCR and mitochondrial respiration with high-resolution respirometry.

Results: In total, 224 children, 11±5 years old (31% female, 65% male) with severe burns encompassing 55±17% TBSA were studied. Gene expression after burn showed that CIDEA PRDM16, TBX1, UCP1, UCP2 and UCP4 increased gradually, CITED, FGF21 and UCP3 decreased, and ZIC1 and TMEM26 fluctuated. REE levels increased significantly in the Early group (1-45 days post burn) and then almost reached healthy levels 1 year after burn. Leak respiration (State 4o) raised to its peak 1 month after burn and then decreased gradually reaching the same levels as the first week.

Conclusion: Browning caused by adrenergic stress after burn injury is naturally reversible when the stimulant seize to exist. Understanding the underlying mechanisms may be useful in clinical practice, for example in the treatment of obese and burned patients.

Key-words: brown adipose tissue, beige, plasticity, adrenergic, mitochondria

3. Abbreviations

BAT	Brown Adipose Tissue
WAT	White Adipose Tissue
UCP	Uncoupling Protein
BMP	Bone Morphogenetic Protein
SNS	Sympathetic Nervous System
FGF	Fibroblast Growth Factor
ANP	Atrial Natriuretic Peptide
BNP	Brain Natriuretic Peptide or b-type Natriuretic Peptide
REE	Resting Energy Expenditure
TBSA	Total Burn Surface Area
PCR	Polymerase Chain Reaction
BMI	Body Mass Index
Myf5	Myogenic factor 5
PGC1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PTH-rP	Parathyroid hormone related protein

Introduction

Currently, adipocytes are divided into two types according to their ability to store energy or produce heat. On one hand, are the unilocular white adipocytes which are the fundamental units of energy storing fat tissue in most animals, and on the other hand two types of thermogenic adipocytes: the highly specialized classical brown and the beige or brite adipocytes. The latter is an intermediate type of adipose tissue between the white and the brown adipose tissue, meaning that it has a brown-like morphology while located into white fat depots.(Seale & Lazar, 2009; Sharp et al., 2012a; Yoneshiro et al., 2013)

Functional and morphological characteristics of brown adipose tissue

Generally, adipose tissue is consisted by white and brown adipocytes with both of them being able to accumulate lipid droplets intracellularly. The main role of the well-known white adipose tissue (WAT) is to store energy mainly as triglycerides and to release fatty acids in the mainstream during fasting periods, a function which helped humans over the years to survive for longer periods between meals. WAT is the main adipose tissue in the human body and is associated with the onset of obesity. Morphologically, white adipocytes are composed by a small number of mitochondria, a peripherally located nucleus and a large spherical lipid vacuole that functions as a storage droplet. Each adipocyte can increase in size and expand its volume by several times. Additionally, WAT functions as an endocrine organ, secreting hormones such as leptin and adiponectin, while participates in the regulation of insulin sensitivity.

Brown adipose tissue (BAT) is a specialized fat tissue that uses energy substrates to produce heat and plays an important role in the regulation of energy balance. Brown fat pads were shown to be highly vascularized and metabolically active, while cause enhanced energy expenditure upon stimulation. Morphologically, brown adipocytes are characterized by multiple, small lipid droplets with a nucleus located in the center of the cell and a high number of mitochondria. BAT produces heat through the action of uncoupling protein-1 (UCP-1) which is densely packed within the mitochondria. When activated, UCP1 uncouples the electron transport across the inner mitochondrial membrane in the respiratory chain from generation of adenosine triphosphate, to release the stored energy as heat. As such, UCP1, which is characteristically abundant in BAT, facilitates the conversion of chemical energy, mainly originating from fatty acids, into thermal energy, producing heat and leading to thermogenesis. Brown adipocytes are gathered at specific homogenous depots located in the interscapular and perirenal regions of rodents where they are richly innervated and vascularized. (Bartness, Vaughan, & Song, 2010)

Brown adipose tissue in adult humans

Studies conducted over the years, supported that BAT may be important for other reasons except from regulating body temperature during cold exposure, such as affecting body weight, lipid and glucose metabolism. Most of these studies used rodents to investigate the physiology and activity of BAT, thus established the presence of BAT and revealed the benefits of activating BAT to regulate body weight. It was observed that "cafeteria-fed" animals had increased mass and activity of BAT, while surgical removal of BAT in mice caused body weight increase and transgenic mice overexpressing UCP1 seemed to be protected against obesity. Additionally, stimulation of beta3-adrenoreceptor resulted in development of brown adipocytes in WAT depots together with an increase in UCP1 expression and reduction of body weight. Even though BAT was known to have all the aforementioned roles, it was still uncertain whether it exists in adult humans.

Among humans, it was known that only newborns and young children have significant brown fat depots, probably to provide sufficient heat in the cold environment following birth, when other ways of producing heat, such as skeletal muscle shivering thermogenesis, have not developed yet. Even though children have a high amount of active BAT, its' volume and activity reduces rapidly after puberty. Adult humans, however, were known to lack brown fat unless specifically challenged by chronic cold or by situations of excessive catecholaminergic concentrations. (CANNON & Nedergaard, 2004a) The first case was observed in 1981, when a study conducted on Finland outdoor workers found that they possess more evident BAT depots surrounding their neck arteries when compared with indoor workers, suggesting that chronic expose to cold temperatures can preserve BAT. (Huttunen, Hirvonen, & Kinnula, 1981) The second case was supported by studying patients having pheochromocytoma which is a catecholamine-secreting tumor located in the adrenal medulla. The occurrence of pheochromocytoma was shown to be associated with activation of BAT in the 1980s, suggesting that human BAT is capable of producing heat and probably contribute to weight loss due to the high levels of catecholamines. (Lean, James, Jennings, & Trayhurn, 1986) Increased secretion of catecholamines can induce weight loss by following multiple pathways. However, it has recently been observed that resection of pheochromocytoma tumor almost eliminated FDG-PET uptake from BAT. (Kuji, Imabayashi, Minagawa, Matsuda, & Miyauchi, 2008) Another study found a correlation between elevated plasma levels of metanephrine and BAT activity, a proof of evidence of the role of catecholamines on stimulation of BAT thermogenesis while an adrenal tumor is present and their negative association with adiposity. (Q. Wang et al., 2011)

The following years, in the 1990s, an incidental finding renewed the scientific interest about BAT. During the usual procedure to detect possible metastasis in cancer patients by

measuring glucose uptake with the FDG-PET scans, radiologists observed a recurrent pattern of bilateral symmetrical glucose uptake in neck regions and in the upper chest which, at first, ascribed to technique malfunction or to muscle activity due to their symmetric distribution. (Engel et al., 1996) Also, in 2002, the use of hybrid PET/CT imaging, proposed that the symmetrical FDG uptake was associated with the adipose tissue and possibly signified BAT activated during the scan due to increased sympathetic activity induced by cold exposure in the imaging room. (Hany et al., 2002)

After several years, new studies highlighted the metabolic significance of BAT in human physiology and its potential role against obesity. (Nedergaard, Bengtsson, & Cannon, 2007) In 2009, a retrospective analysis of FDG-PET/CT scans performed in 1972 on various patients, was able to identify depots of BAT in the anterior neck and thorax. (Cypess et al., 2009a) It was calculated that the probability of BAT detection was inversely correlated with age, use of beta-blocker, outdoor temperature and body mass index in older patients. It must be also noted that UCP1 activity was found in 33 biopsies from the same cervical and supraclavicular regions in which BAT was observed on PET/CT. The next step was to examine BAT activity when subjects were intentionally exposed to cold. Mild exposure to cold, meaning a 2 hour exposure to 15°C while ensuring that cold-induced shivering thermogenesis by muscles was not activated, led to detection of BAT activity using FDG-PET/CT in 23 out of 24 healthy men. Once again, BAT activity was inversely correlated with BMI supporting the notion that BAT may be used for the treatment of obesity. (Wouter D. van Marken Lichtenbelt et al., 2009a) Another study observed a significant difference of BAT activity levels between young and older participants, with the former having more evident cold-induced uptake of FDG-PET/CT. (Saito et al., 2009) Additionally, Virtanen et al. showed that cold exposure increased by 15-fold the FDG-PET/CT uptake in the supraclavicular area of 5 healthy participants. (Virtanen et al., 2009a) Tissue biopsies of areas having enhanced glucose uptake gathered from three of those participants and found to have mRNA for BAT markers, and substantial levels of UCP1 and cytochrome c (a mitochondrial marker abundant in BAT).

In order to study the metabolic activity of BAT, blood perfusion was measured using intravenous injections of $[^{15}O]H_2O$ while performing PET/CT emission scan. It was found that blood perfusion increase was more than 2-fold and glucose uptake was also increased, when cold-stimulated, suggesting that BAT is highly vascularized and has high oxygen demands. (Orava et al., 2011) Another study showed that cold-activated BAT is characterized by higher oxidative metabolism. (Ouellet et al., 2012) In this study radioactive acetate was used, which is distributed to tissues depending on their blood flow and loss of radioactivity is an indication of active oxidative metabolism of the tissue. As such, radioactivity was lost in activated BAT while

remained stable in thermoneutral subjects. Additionally, nonesterified fatty acid uptake was increased in activated supraclavicular BAT showing that triglycerides may be used as energy substrates for BAT thermogenesis.

It was only recently that the presence of significant depots of genuine brown fat in adult humans was proven based upon radiological observations of symmetrical [18F]-2-fluoro-D-2-deoxy-D-glucose (FDG) positron emission tomography (PET)-positive loci in the supraclavicular and spinal regions. These regions were subsequently confirmed by biopsy to contain bona fide UCP-1^{+} adipose tissue consistent with brown fat. (Cypess et al., 2009b; Wouter D. van Marken Lichtenbelt et al., 2009b; Virtanen et al., 2009b) The physiological role of BAT's location in the trunk, the supraclavicular and neck regions, might be to provide warm blood to the brain.

The process leading to the activation of BAT and production of heat is mainly controlled by the sympathetic nervous system, which increases the glucose and fatty acid uptake from BAT during adrenergic response. This thermogenic activity is mediated, in particular, by the hypothalamus and is regulated by a wide range of transcriptional factors and regulators.

BAT origins

At first, the fact that brown and white adipocytes share common morphological characteristics and both store triglycerides in intracellular lipid droplets, supported the notion that they have common origin. However, later studies proved that WAT and BAT have different developmental origins. Brown adipocytes can develop from two different pathways. On one hand, classic brown adipocytes located at typical brown fat pads, develop from muscle progenitor cells, which express myogenic factors such as myf5. Myf5 acts as regulatory factor playing an important role in myogenesis and it was thought to be expressed only in precursor cells of skeletal muscle. (PRDM16 controls a brown fat/skeletal muscle switch) Expression of myf5 found to be important in order to distinguish classic BAT from recruitable (beige) BAT, which is negative for the myf5 marker and is also dispersed in WAT depots. The origins of recruitable BAT will be analyzed later in the text. The fact that classical brown and beige adipocytes develop from different progenitor cells suggests that they might respond differently to physiological and environmental signals and therefore represent two separate targets for pharmacological intervention.

Beige adipocytes

For many years, beige (brite) adipocytes, were poorly characterized and simply called brown adipocytes while having an overlapping but distinct gene expression pattern compared with the brown adipocytes. Beige adipocytes are scattered into white adipose tissue and develop in

response to certain environmental cues, such as chronic cold exposure, a process usually named "browning" of white adipose tissue. Their abundance varies dramatically between different body areas, with the highest numbers found in inguinal and retroperitoneal fat, and much lower numbers located in perigonadal fat. Recent studies presented solid proof of the occurrence of browning in adult humans, making beige adipocytes an attractive therapeutic target for obesity and obesity-related diseases, including type 2 diabetes.

Beige adipocytes seem to have common origins with white adipocytes. Beige fat can be developed through either transdifferentiation of WAT into BAT-like adipocytes following appropriate stimulation, such as adrenergic stress and high fat diet, or alternatively by development from early bipotential adipocyte progenitors located in the perivascular region of WAT. (Y.-H. Lee, Petkova, Mottillo, & Granneman, 2012) Accordingly, they share some common molecular and functional characteristics. More specifically, they are reproducibly highly adipogenic and have similar levels of adipogenesis and expression of markers specific to fat cells, such as *Adiponectin* and *Pparγ*. (Wu et al., 2012) Accordingly, they seem to have low basal levels of gene expression for *Ucp1, Cidea* and *Cox7a1* in comparison with the levels observed in classical brown fat.

Brown vs. Beige adipose tissue

The similarities and differences between brown and beige adipose cells, as well as their relative importance in energy homeostasis are not totally clarified yet. Both BAT and WAT express a core program of thermogenic and mitochondrial genes, including UCP-1, thus are truly thermogenic tissues, with a large fraction of their respiration being uncoupled. When comparing pure clonal brown and beige cells, it appears that the classical brown fat cells have a higher basal UCP-1 expression and increased uncoupled respiration (relative to white or beige cells) before hormonal stimulation. Beige cells, on the other hand, have low basal UCP-1 expression and uncoupled respiration, comparable to brown cells. However, stimulation with a β-adrenergic agonist elevates UCP-1 expression in beige cells reaching levels similar to those of brown fat cells. This observation suggests that beige cells are uniquely programmed to be bifunctional, meaning that are capable of storing energy in the absence of thermogenic stimuli but also fully capable of increasing heat production when appropriate signals are received (Wu et al., 2012). It must be noted that selective loss of classic BAT causes compensatory activation of beige fat, restoring both body temperature and resistance to diet-induced obesity, suggesting significant overlap in function. (Tim J. Schulz et al., 2013a) Additionally, regarding their concentration in different locations in adult humans, it has been found that the ratio of brown:beige fluctuates according to

the specific depot sampled and increases as one moves deeper within the neck and back (Jespersen et al., 2013; Lidell et al., 2013; Tim J. Schulz et al., 2013a; Sharp et al., 2012b; Wu et al., 2012).

Browning

The transdifferentiation between white and beige adipocytes was observed decades ago, when it was found that a subset of traditionally white adipose cells in the parametrial adipose depots, adopted a brown-like morphology in mice exposed to cold temperatures, characterized by small multilocular cells enriched with numerous mitochondria. As such, the aforementioned process was named "browning" and characterizes the switch of white adipocytes into beige when specific stimulants occur.(Cohen et al., 2014a; M. Harms & Seale, 2013; Shabalina et al., 2013)

The plasticity of adipose tissue has already been observed in animal models. In 1991, brite adipocytes were characterized as "convertible adipose tissue". More specifically, the aforementioned published work on mice used cold exposure as a stressor for 1 week which resulted in a significant increase of UCP and lipoprotein lipase mRNA in inguinal adipose tissue samples, to an extent resembling classical brown adipocytes. The same mice were rewarmed at 28 ^{0}C and their inguinal adipose tissue was analyzed in comparison with interscapular brown adipose tissue and epididymal white adipose tissue for another 37 days. During that time, inguinal adipocytes ceased expressing UCP mRNA; mitochondria of inguinal adipocytes rich in UCP were destroyed and replaced with common mitochondria; and UCP was undetectable immunohistochemically. Adipocytes accumulated lipids, and the tissue morphologically once again resembled white adipose tissue.(Loncar, 1991) Later, Rosenwald et al., induced the formation of brite cells by exposing mice to chronic cold, a process reversed by a 5-week warm adaptation. Although, the brite adipocytes formed by cold stimulation were not eliminated. Genetic tracing and transcriptional characterization of isolated adipocytes demonstrated that they were converted into cells with the morphology and gene expression pattern of white adipocytes. (Rosenwald, Perdikari, Rülicke, & Wolfrum, 2013)

The processes of whitening and browning have been studied in different biological models to assess the impact of different stimuli on the transdifferentiation between white and beige adipocytes. However, there is a lack of literature concerning the browning of WAT in humans. The methodology used to study browning, included histological (number and size of multilocular adipocytes), molecular (quantification of UCP1 and other markers), immuno-histochemical and metabolic analyses (measurement of energy expenditure). From the human studies, three have recently discovered the role of WAT browning in the development and progression of hypermetabolism in cancer and burn patients.(Patsouris et al., 2015; Petruzzelli et al., 2014a; L.

Sidossis et al., 2015) In the first study, on cachexic cancer patients, Wagner et al. found a phenotypic switch from WAT to BAT. In the second and third studies, Herndon et al. found a similar phenotypic shift of WAT to a more brown-like phenotype in postburn pediatric patients.(L. Sidossis et al., 2015) Patsouris et al. confirmed these findings in adult burn patients, as well as postburn mice.(Patsouris et al., 2015) Generally, these studies proved the occurrence of WAT browning in humans, independent from the pathological conditions. The next step, was the determination of the metabolic pathways which regulate whitening and browning, and identify factors that affect them.

Stimulation of browning by external factors

Sympathetic Nervous System

The Sympathetic Nervous System (SNS), with several hypothalamic and extrahypothalamic areas plays an important role as integrator of cold response, by regulating dynamic changes as well as prolonged adaptation. (CANNON & Nedergaard, 2004b) During cold exposure, norepinephrine is released by sympathetic nerves and bind with adrenergic receptors in BAT leading to the activation of adenylate cyclase to increase intracellular cAMP levels, which then activate PKA (cAMP-dependent protein kinase). Then, PKA phosphorylates hormone-sensitive lipase causing the hydrolysis of TAG into NEFAs, the main substrate for heat production in BAT. Long-chain NEFAs increase UCP1 activity which leads to higher levels of uncoupling mitochondrial oxidation and amplified heat production. (CANNON & Nedergaard, 2004b)

Due to the effect of sympathetic nervous system on BAT stimulation, drugs that increase sympathetic nerve activity, such as sibutramine and ephedrine, have been studied to determine their role on thermogenesis, metabolic rate and weight control. Although, it was found that long term use of beta3-adrenergic agonists is dangerous for the cardiovascular system, because they increase the risk for vascular events.(Larsen et al., 2002; Redman et al., 2007)

Cold exposure

As mentioned above, cold exposure causes increase of the thermogenic activity of BAT, controlled by the SNS. In addition, these pathways can also robustly stimulate UCP1 expression through the phosphorylation of key transcriptional activators, such as activating transcription factor 2 (ATF2), PPARγ coactivator 1α (PGC1α), and cAMP response element–binding protein (CREB). Although, it is remarkable that cold exposure can affect browning independently of SNS signaling. That is possible because white and beige (but not brown) adipocytes can directly sense temperature changes. A proof of this capability is the browning of white fat in mice lacking all β-adrenergic receptors and having diminished thermogenic gene induction in interscapular BAT after cold exposure. Additionally, a large number of circulating hormones seems to be implicated

with BAT activation during cold exposure. Some of these hormones are triiodothyronine (T3), hepatic bile acids, FGF21, cardiac hormones (such as ANP, BNP and cardiotrophin-1) and irisin (produced by skeletal muscle in response to exercise) (Boström et al., 2012a; Villarroya & Vidal-Puig, 2013).

Exercise

Studies on animal models using exercise protocols with different frequencies showed that exercise is able to induce WAT browning and increase energy expenditure. (Boström et al., 2012b; Xu et al., 2011) The molecules that are involved in the browning process when exercising include the transcriptional coregulator PGC1a which upregulates two novel myokines, meteorin-like (MTRNL) and irisin. Additionally, a major myokine, IL-6, has been shown to stimulate the development of beige adipocytes and also found to be necessary for exercise induced WAT browning in mice.(Knudsen et al., 2014) Interestingly, lactate, which is mainly produced in skeletal muscles during exercise due to anaerobic glycolysis, has been shown to promote WAT browning.(Carriere et al., 2014) The browning process induced by lactate is mediated by a change in intracellular redox state (NADH-to-NAD+ ratio) by the monocarboxylate transporters that carry lactate into the cells. However, the physiological significance of lactate on exercise-induced WAT browning needs to be studied further.

Cancer cachexia

The main characteristics of cachexia are severe weight loss due to muscle and adipose atrophy, and chronic inflammation. Patients with cancer cachexia have been found to develop WAT browning and increased energy expenditure. In order to identify the mechanism behind this observation, two techniques were used: an IL-6–deficient tumor was implanted in mice with cancer cachexia, and pharmacological approaches using an anti-IL-6 monoclonal antibody, a β3-adrenergic receptor antagonist and the nonsteroidal anti-inflammatory drug sulindac. All these interventions resulted in lower WAT browning and severity of cachexia, indicating that IL-6 acts as a major mediator of cachexia-induced beige adipocyte development.(Petruzzelli et al., 2014b) Additionally, tumor-derived parathyroid hormone–related protein (PTHrP) was found to stimulate WAT browning and energy expenditure in patients with Lewis lung carcinoma.(Kir et al., 2014a) Similarly, neutralization of PTHrP in mice, blocks WAT browning and protects mice from the symptoms of cachexia leading to preservation of adipose and muscle mass.

Stimulation of browning by internal factors

Catecholamines

In burned patients, marked increases of catecholamine levels have been noted even years after the initial injury. (Kulp, Herndon, Lee, Suman, & Jeschke, 2010) This sustained increase of catecholamines in burned patients has recently been shown to stimulate WAT browning and the sequence of events leading to the hypermetabolic response.(Patsouris et al., 2015; L. Sidossis et al., 2015) Initially, it was hypothesized that only adrenal glands can produce catecholamines, however more recent studies showed that macrophages can produce catecholamines too. It was shown that when activated, polarized M2 macrophages are recruited in subcutaneous WAT and secrete catecholamines to promote WAT browning. (Abdullahi & Jeschke, 2016) The effect of macrophages on browning was initially found to depend on interleukin 4 (IL-4) signaling, as mice missing IL-4 signaling exhibited impairments in the browning process. (Abdullahi & Jeschke, 2016) However, resent research showed that palmitate, which is an abundant FFA in the serum of burn patients, has also the ability to regulate macrophage polarization. (Jeschke et al., 2015; Xiu, Catapano, Diao, Stanojcic, & Jeschke, 2015; Xiu, Diao, Qi, Catapano, & Jeschke, 2016) This chemical communication between macrophages and adipose tissue is intriguing as it may imply a loop, as WAT browning induces lipolysis and increases FFA blood levels which in turn polarize macrophages, therefore sustaining WAT browning during the hypermetabolic response. (Abdullahi & Jeschke, 2016)

Interleukin-6

When it was discovered, interleukin 6 (IL-6) has been characterized mainly by its pro-inflammatory and immunological roles during body's response to infection and injury. (Schett, Elewaut, McInnes, Dayer, & Neurath, 2013) Now it is mostly considered as a cytokine which also has hormone-like properties which affect energy and glucose balance. (Jones et al., 2011; Rohleder, Aringer, & Boentert, 2012) Also, there is an increasing notion that wants IL-6 to exert a profound impact on the browning of WAT. For example, an animal study showed that mice missing the IL-6 gene have impaired WAT browning in response to burn injury. Other research findings support this observation, as continued activation of IL-6 signaling promotes WAT browning and increases energy expenditure in cancer cases. (Petruzzelli et al., 2014a) Eosinophils regulate IL-4 and IL-13 signaling to stimulate alternatively activated (type 2/M2) macrophages in the subcutaneous WAT and secrete catecholamines to activate WAT browning. (Mauer et al., 2014; Nguyen et al., 2011; Qiu et al., 2014) Although the involvement of catecholamines and IL-6 in WAT browning is now undeniable, the details surrounding this process remain unknown.

Parathyroid-Hormone-Related Protein

During the past years, parathyroid-hormone-related protein (PTH-rP) has been known for its beneficial effects on skin, development of bones, cartilage and placenta. (Guntur, Doucette, & Rosen, 2015; Maioli, Fortino, Torricelli, Arezzini, & Gardi, 2002; W. Sun et al., 2016) However, a recent study on humans and rodents, has implicated PTH-rP in promoting browning of WAT during cancer. (Kir et al., 2014b) More specifically, PTH-rP secreted from the tumor to the bloodstream has been suggested to promote the browning process. Unrevealing the signaling pathway that takes place during hypermetabolic responses will be the foundation of medicinal utilization of agents that mark PTH-rP-induced thermogenesis.

Meteorin-like

Meteorin-like (METRNL), another novel myokine, is upregulated by the exercise induced PGC1α pathway and activate beige adipocyte development.(Boström et al., 2012c; Rao et al., 2014) METRNL promotes an eosinophil-dependent activation of M2 macrophages and induces WAT browning.

Irisin

In 2012, irisin was identified as a muscle tissue secreted peptide, recommending a possible mechanism by which exercise increases browning.(Boström et al., 2012d) Irisin induces the expression of specific beige-selective genes in a cell-autonomous manner. More specifically, when humans and rodents exercise, PGC1α levels increase in the muscles leading to increased fibronectin type III domain containing protein 5 (FNDC5) which is further cleaved to irisin. (Baar et al., 2002; Goto et al., 2000) Irisin, in turn, can affect the thermogenic activity of different tissues, such as BAT, by increasing UCP1 expression.(Boström et al., 2012a; Zhang et al., 2014) Irisin also induces browning by promoting the expression of UCP1 and other BAT-specific genes in sWAT through mitogen-activated protein p38 MAP and ERK MAP kinase signaling, while genes characteristic of WAT are less expressed.(Baar et al., 2002; Zhang et al., 2014) More recently, one report showed that the actions of irisin might be of clinical value since both irisin and FGF are cold-induced endocrine activators of BAT activity in humans.(P. Lee et al., 2014) However, there are also several studies that have shown controversial results, and some authors failed to document an effect by contraction in circulating irisin levels in humans or an effect on beige/brite differentiation of human preadipocytes.(Pekkala et al., 2013; Raschke et al., 2013) Therefore, the potential beneficial metabolic actions of irisin during exercise are still under debate, and some issues await clarification. These include the full characterization of the different tissues expressing irisin and, more importantly, the different proteolytic mechanisms involved in its post-translational

processing and the generation of putative secreted molecules.(Novelle, Contreras, Romero-Picó, López, & Diéguez, 2013; Roca-Rivada et al., 2013) As such, it seems that further studies are needed to clarify, in depth, this field.

Endocrine hormones and metabolites

Several endocrine factors have been identified for being able to increase energy expenditure, protect animals from diet-induced body weight gain, and control glucose homeostasis and/or insulin sensitivity, such as BMP4, -7, -8b, FGF19, -21, GDF5, ANP, prostaglandins, VEGF and BAIBA. Others are known for being negative regulators of the aforementioned and observed to have increased expression under obesity conditions (TGFβ, Aldh1, etc.).

Bone morphogenic proteins (BMPs)

Bone morphogenic proteins (BMPs) were originally known for their role in inducing bone formation, but later it was found to be involved in the development and function of many tissues, such as the intestine, brain and WAT.(Hogan, 1996) Energy homeostasis and early steps of adipogenesis are affected by some types of the BMP family. Forced expression of BMP4 in WAT of mice stimulates the production of beige adipocytes while leading to decreased WAT mass and lower risk for developing its metabolic complications.(Qian et al., 2013) In addition to these changes increased energy expenditure, improved insulin sensitivity, and protection against diet-induced obesity and diabetes was profound in these animal models. On the contrary, BMP4-deficient mice exhibit higher WAT mass and impaired insulin sensitivity. PGC1α was found to be the target of BMP signaling required for these browning changes in WAT. These effects of BMP4 on WAT seem to extend to human adipose tissue as well as the expression of BMP4 in WAT is inversely associated with body mass index. However, some studies showed that BMP4 promotes the differentiation of mesenchymal stem cells into white adipocytes, encouraging fat storage and decreasing energy expenditure in rodents.(Contreras et al., 2015; Modica & Wolfrum, 2013) Thus, the relationship between BMP4 and browning seems to differ depending on the model used for each study.

BMP7 has been associated with the development of BAT, being able to drive the complete brown fat differentiation program, including PRDM16 expression.(Modica & Wolfrum, 2013) BMP7 seems to be able to stimulate a subpopulation of adipogenic progenitors, from skeletal muscle and subcutaneous white fat of mice, to differentiate into beige adipocytes.(T. J. Schulz et al., 2011) Similarly, human preadipocytes isolated from subcutaneous white fat were more likely to transform into beige adipocytes compared with cells isolated from mesenteric or omental white fat. Interestingly, BMP7 has the unique ability to stimulate the differentiation of brown

preadipocytes even in the absence of the usually required hormones. BMP7 can also alter energy homeostasis by affecting the expression of UCP1, regulators of brown fat development, such as PRDM16 and PGC-1α, adipogenic transcription factors PPARγ and C/EBPs, and the mitochondrial biogenesis controlled by p38MAPK (Mapk14) and PGC1.(Tseng et al., 2008) As a result of its effect on BAT, BMP-7 was also reported to improve insulin sensitivity.(T. J. Schulz et al., 2011) It must be noted that hypothalamus was found to express BMP7, suggesting that BMP7 may regulate browning thru a central mechanism who is also responsible for reduced food intake.(Modica & Wolfrum, 2013)

BMP8B is able to regulate thermogenesis directly and it was found to be expressed in both BAT and the hypothalamus.(Contreras et al., 2015; Whittle et al., 2012) In mature BAT is induced by nutritional and thermogenic factors leading to increased response to noradrenaline through enhanced p38MAPK/CREB signaling and increased lipase activity. Central administration of BMP8B increased thermogenesis and core temperature, leading to weight loss.(Contreras et al., 2015) This effect, was mediated by the ventromedial hypothalamus (VMH) though an AMPK-dependent pathway, without any change in the feeding behavior.(Contreras et al., 2015; Whittle et al., 2012) Additionally, Bmp8b deprived mice exhibited impaired thermogenesis and reduced metabolic rate, causing weight gain despite the limited access to food. It was also observed that these mice displayed altered neuropeptide levels and reduced phosphorylation of AMPK, indicating an anorexigenic state. Central injection with BMP8B increased the sympathetic activation of BAT, depending on the AMPK levels in key hypothalamic nuclei. As such, it seems that BMP8B may offer a possible mechanism to explicitly increase heat production by BAT.

FGFs (FGF19, -21)

Fibroblast growth factor 21 is a member of the fibroblast growth factor (FGF) family that is expressed in BAT and WAT, but mainly in the liver, and acts as an endocrine hormone to regulate carbohydrate and lipid homeostasis as well as body weight.(80–85) Expression of FGF21 is regulated differently in each tissue during chronic cold exposure in mice, meaning that its' expression increases in WAT and BAT, but decreases in the liver.(f. M. Fisher et al., 2012a) It was found that the increased expression in WAT leads to significant elevation of UCP1 expression, thus leading to browning of subcutaneous tissue. Notably, in humans, a mild cold exposure (12h to 19°C) was shown to increase plasma FGF21 levels, which was positively correlated with the changes in adipose tissue glycerol and total energy expenditure.(P. Lee et al., 2013) This may display that FGF21 plays a comparable role in humans as in rodents in controlling cold-induced metabolic changes. Another study used LIRKO mice having deactivated the liver-specific insulin

receptor which exhibit severe insulin resistance, glucose intolerance, and a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression.(Emanuelli et al., 2014a) When those mice were treated with FGFR21 hyperglycemia was completely normalized, even though FGF21 did not reduce gluconeogenesis. The control of blood sugar was improved partially due to increased glucose uptake from brown fat, browning of white fat, and overall increased energy expenditure. Although, under these conditions, FGF21 was not able to improve lipid metabolism. Thus, FGF21 seems to improve glycemic control in diabetic mice independently of insulin action in the liver by increasing energy metabolism through activation of browning. It seems that PPARγ is involved with the secretion of FGF21 in adipose tissue by transcriptionally controlling FGF21, which in turn acts in an autocrine or paracrine manner to increase PPARγ transcriptional activity, independently of mRNA expression, in a feed-forward loop system.(Dutchak et al., 2012; H. Wang, Qiang, & Farmer, 2008) Mice deficient in FGF21 were found to be less capable to adapt to long-term cold exposure because of limited browning.(F. M. Fisher et al., 2012) In the contrary, systemic administration of FGF21 in obese mice resulted in reduced adiposity, improved glycemic control, as well as increased energy expenditure.(Coskun et al., 2008) The aforementioned evidence suggest that FGF21 plays a significant role in glucose metabolism by regulating UCP1 expression.

Accordingly, it was observed that FGF19 transgenic mice had a significant decrease of adipose tissue that caused by an increase in energy expenditure.(Tomlinson et al., 2002) Moreover, these mice did not become obese or diabetic when followed a high fat diet.(Fu et al., 2004) The FGF19 transgenic mice had higher brown adipose tissue mass and reduced expression of acetyl coenzyme A carboxylase 2 in liver together with lower expression of the leptin receptor, suggesting two mechanisms by which FGF19 may increase energy expenditure. In order to clarify the underlying mechanisms leading to increased metabolic rate, the gene expression changes caused by FGF19 treatment, in BAT and the liver, were analyzed.(Fu et al., 2004) In BAT, it was found that chronic exposure to FGF19 stimulated a gene expression program which leads to the activation of this tissue. The gene expression changes in liver supported the experimentally determined increase in fat oxidation (decreased respiratory quotient) and decrease in liver triglycerides. As such, FGF19 is capable of increasing the metabolic rate concurrently with an increase in fatty acid oxidation.

GDF5 (Growth Differentiation Factor 5)

Originally, GDF5 was known for its role in skeletal development and joint morphogenesis in mammals. However, later, it was found that it has an important part in regulating brown

adipogenesis.(Pei, Yang, Kiess, Sun, & Luo, 2014) Transgenic overexpression of GDF5 in mice adipose tissues can stimulate browning, expression of UCP1 in inguinal subcutaneous WAT, and energy expenditure, leading to a leaner phenotype with lower susceptibility to diet-induced obesity. On the contrary, depletion of GDF5 in mice resulted in a significant impaired energy expenditure and thermogenesis. The underlying mechanisms involve bone morphogenetic protein receptor (BMPR), PGC-1α and the decapentaplegic homolog (Smad). These observations suggest that browning and energy homeostasis are both positively regulated by expression of GDF5 in adipose tissues.

Natriuretic Peptides

At first, cardiac natriuretic peptides were known for stimulating lipolysis in human adipocytes.(Pei et al., 2014) Later studies showed that they are capable of stimulating browning and thermogenic energy expenditure mediated by UCP1, using a pathway involving PGC1α. Interestingly, at low concentrations, ANP and β-adrenergic agonists additively enhanced the expression of brown fat and mitochondrial markers. These results suggest that the heart acts as a central regulator of adipose tissue development.

VEGF (Vascular endothelial growth factor)

During obesity fat mass expands extensively but vascularization of adipose tissue is insufficient leading to local hypoxia which alters adipokine expression, proinflammatory macrophage recruitment, and insulin resistance(K. Sun et al., 2012). It was found that local increase of VEGF expression in WAT and BAT improves blood vessel number and size in both adipose tissues and causes browning through UCP1 and PGC1α increase.(Elias et al., 2012) These changes increased thermogenesis, energy expenditure, whole-body insulin sensitivity and glucose tolerance.

Prostaglandins (such as Cox2)

Cyclooxygenase (COX2), a rate-limiting enzyme in the synthesis of prostaglandins, found to be crucial for the beta-adrenergic signaling in WAT in order to stimulate browning.(Madsen et al., 2010; Vegiopoulos et al., 2010) COX2 is essential for the increase of UCP1 expression in WAT, but not in classical brown adipocytes.(Madsen et al., 2010) As such, upregulation of COX2 in WAT resulted in increased systemic energy expenditure and also protected the mice against obesity when fed a high-fat diet.

Transcriptional control

Many studies about brown and beige fat are focused on the physiological activators of thermogenesis in these cells. An overview of those activators is shown in Figure 4 (Activators of Beige and Brown adipocytes development and function). Some activators appear to work primarily by inducing the production of new beige (e.g., irisin) or brown (e.g., BMP7) adipocytes, while others may act on both recruitment and enhancement of thermogenic activity.

Browning is positively regulated by PR domain–containing protein 16 (PRDM16) and its binding molecules, such as CCAAT/enhancer-binding protein-β (C/EBPβ), early B cell factor 2 (EBF2), PGC1α, and euchromatic histone-lysine N-methyltransferase 1 (EHMT1). Studies showed that inhibition of these factors disrupts fate determination and/or maintenance of brown and beige adipocytes.(M. J. Harms et al., 2014; Rajakumari et al., 2013; Seale et al., 2008) Accordingly, lack of PRDM16 or EHMT1 significantly affected the browning, caused by cold-exposure and PPARγ agonists.(Cohen et al., 2014b; Ohno, Shinoda, Ohyama, Sharp, & Kajimura, 2013) The transcriptional complex of PRDM16 bind with C/EBP-β acts in myf5-positive myoblastic precursors or pre-adipocytes to induce the expression of PPARγ and PGC-1α, which activate the browning process.

FGF21 belongs to a group of endocrine factors that control the development of brown and beige adipocytes. As such, they have the capability to increase whole-body energy expenditure, keep animals from gaining weight due to diet-induced changes, and improve systemic glucose homeostasis and/or insulin sensitivity. (Emanuelli et al., 2014b; f. M. Fisher et al., 2012b; P. Lee et al., 2014) More specifically, it has been observed that FGF21 increases during cold-exposure resulting to increased energy expenditure, independent of fat and lean mass, gender, and age. (P. Lee et al., 2013)

Clinical significance of BAT

BAT against obesity

Several animal studies showed the benefits of functional BAT on the regulation of body weight and the onset of adipocity. The derived observations, included the increase of BAT mass and activity in “cafeteria-fed” animals, increase of body weight in mice after surgical excision of BAT, protection against obesity in transgenic mice overexpressing UCP1, formation of brown adipocytes in WAT regions, UCP1 overexpression and body weight reduction after adrenergic stimulation of the beta3-adrenoreceptor.

The unique characteristics of BAT, managing the use of a large amount of circulating lipids to facilitate thermogenesis, produce heat and modify energy expenditure, mark it as a potential

therapeutic target in obese subjects. Thus, an elegant approach to reduce body fat might involve the preferential activation of brown or beige adipocytes, a strategy that many investigators focused on. Based on the metabolic rate of BAT in mice (about 300 W/kg, approximately two orders of magnitude higher than any other tissue), it was calculated that 40-50g of BAT could account for 20% of daily energy expenditure, which would be remarkable given current estimates that there may be upward of 100g of BAT in a normal human (Cannon and Nedergaard, 2004, Rothwell and Stock, 1979, van Marken Lichtenbelt et al., 2009). Although, this claim is based on two major assumptions which have not been proved yet. First, the mouse calculations for BAT were made under conditions of maximal activation, which rarely happens in humans trying to achieve thermoneutrality. Second, mammalian energy expenditure is inversely correlated with body size, which in that case means that whole-body BMR is 1-2W/kg for humans was compared with 8W/kg for mice (W. D. van Marken Lichtenbelt & Schrauwen, 2011). Even with these limitations, BAT activity has been predicted to account for 2.7%-5% of BMR in humans, which could cumulatively promote more than 4kg of fat loss per year (W. D. van Marken Lichtenbelt & Schrauwen, 2011; Virtanen et al., 2009b).

An environmental factor suggested to play a role in energy balance and could explain the role of BAT on the onset of obesity, is the reduced variability of ambient temperature during the day and mostly reduced exposure to seasonal cold. Cheap and efficient energy sources, extensive use of air conditioning and heating, reduction of outdoor activities and increased time spent indoors are the main causes of reduced exposure to seasonal temperature variabilities. This "thermal comfort" zone is suggested to lessen energy expenditure and the need for physiological thermogenesis and thus contribute to the increased prevalence of obesity in the population.(Johnson, Mavrogianni, Ucci, Vidal-Puig, & Wardle, 2011)

Hypothesis and aim

The hypothesis of the current study was that whitening process occurs in burned patients after the browning process. Also, we aimed to investigate how much time post-burn, the whitening process happens.

4. Methodology

Burn patients

Children with burns admitted to Shriners hospitals for Children - Galveston between 2012 and 2015, encompassing more than 30% of their total body surface area (TBSA) were included in the current study, which was approved by the Institutional Review Board at the University of Texas Medical Branch. All burn patients received standard burn care after admission to the hospital, which included fluid resuscitation, excision of all full-thickness burn wounds, and auto-grafting to close burn wounds. Prior to participation in the study, informed written consent was obtained from the parent or legal guardian of each burned child. Throughout this acute hospitalization, patients were fed 1500 kcal/m2 body area plus 1500 kcal/m2 burned body area of a total enteral nutrition formula (82% carbohydrates, 3% fat, 15% protein) via a nasogastric feeding tube (Vivonex T.E.N., Nestlé Health Science, Minneapolis, MN). Cohorts of burn patients were studied during the acute hospitalization period and after discharge when they returned to the hospital for follow-up care. On each occasion, indirect calorimetry was performed to calculate the degree of hypermetabolism, and a biopsy was collected under sedation and local anesthesia to determine mitochondrial respiratory capacity and function.

Healthy participants

Healthy control subjects were young adults recruited from the Galveston County of Texas area via local advertisement to serve as healthy unburned controls. These participants were admitted to the Clinical Research Center at the University of Texas Medical Branch. Informed and written consent were obtained before participation in the study. Due to ethical constraints, we were unable to obtain samples from healthy children.

sWAT samples were collected from the torso or limbs of healthy and severely burned children and adults during their scheduled surgeries while they were under general anesthesia. For burn patients, burn wounds were excised down to the healthy adipose tissue to provide a viable wound bed for skin grafting. sWAT samples were taken from the exposed healthy sWAT. sWAT was sampled from the torso or limbs depending on the location of the skin graft procedure.

The study protocol was reviewed and approved by the Institutional Review Board (IRB) of the University of Texas Medical Branch (UTMB), Galveston, Texas.

Resting Energy Expenditure:

Resting energy expenditure (REE) was measured in order to determine the degree of hypermetabolism in burned participants. REE was calculated by respiratory gas exchange (oxygen consumption and carbon dioxide production) measured by using a ventilated hood system (Sensor Medics, Yorba Linda, CA) and was compared to the Harris-Benedict equation.(WEIR, 1949)(Roza & Shizgal, 1984)

REE was calculated with the Weir equation and the measured values of CO2 and VCO2. The Weir equation used was:

{[(3.9*VO2)+(1.1*VCO2)]*1.44}*1000

Gene expression:

Approximately 50-100 mg of adipose tissue was used for RNA extraction using a pure link RNA Isolation Mini Kit Total (Life Technologies, Carlsbad, CA) or RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA), including an on-column DNAse digestion step. Quantification of the isolated RNA was performed using Biotek Take3 micro-volume plate reader (Biotek Instrument, Inc, Vermont). cDNA was produced using High-Capacity RNA-to-cDNA™ Kit (Life Technologies, Carlsbad, CA) and pre-amplified using TaqMan® PreAmp Master Mix Kit (Life Technologies, Carlsbad, CA). Quantitative real-time-PCR analyses were performed on an ABI PRISM 7900HT using the TaqMan® Gene Expression Master Mix (Life Technologies, Carlsbad, CA) with pre-amplified cDNA and specific Taqman gene expression assays (Life Technologies, Carlsbad, CA). The housekeeping gene used to normalize the expression of the target genes was GAPDH. Analyses of the relative gene expression were performed using DataAssist™ Software Version 3.01(Life Technologies) to derive delta-CT values.

High-Resolution Respirometry

A portion of each adipose tissue biopsy was immediately transferred to an ice-cold preservation buffer after collection (10 mM EGTA containing 6.6 mM MgCl2, 50 mM MES, 0.5 mM dithiothreitol, 20 mM taurine, 20 mM imidazole, 5.8 mM ATP, and 15 mM PCr) and stored on ice. Samples were immediately transferred to the laboratory where they were blotted on filter paper and weighed. Thereafter, approximately 25-50 mg of WAT were transferred to Oxygraph respirometer chambers (Oroboros Instruments, Innsbruck, Austria) and suspended in 2 ml of mitochondrial respiration buffer (0.5 mM EGTA buffer containing 20 mM HEPES, 3 mM MgCl2, 10 mM potassium phosphate, 60 mM potassium lactobionate, 20 mM taurine, 110 mM sucrose, and 1 g/l bovine serum albumin). Adipose tissue was permeabilized by the addition of 2μM

digitonin directly into the respirometer chamber, as described previously (Kraunsoe et al., 2010; Porter et al., 2015; Sidossis et al., 2015). Once the O_2 consumption signal had stabilized, a background leak respiratory rate was recorded (basal). Thereafter, substrates (1.5 mM octanoyl-carnitine, 5 mM pyruvate, 10 mM glutamate and 2 mM malate) were titrated into the chamber and State 2 (leak) respiration was recorded. Then, 5 mM ADP was titrated into the Oxygraph chamber to transition to State 3 (coupled) respiration. All measurements were made at 37°C, and O_2 concentration was maintained between 100-300 nmol/ml for all respirometry measurements.

Statistical analysis:

Statistical analysis and graph drawing were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla). All data are presented as mean ± standard error mean (SEM).

5. Results

Patient demographics

Demographics of the included burned patients are presented in Table 1. In total, 244 children 11±5 years old were studied from which 65% were males and 31% were females. Patients had large burns encompassing 55±17%, on average, of the total body surface area (TBSA), 41±23% of which were third degree burn lesions. The data gathered from these patients were assessed to produce the physiological and functional evidence of browning (see "Physiological Evidence" and "Functional Evidence").

	Total (n=244)	**1 week (n=65)**	**1 month (n=62)**	**3 months (n=31)**	**6 months (n=45)**	**1 year (n=41)**
Age (years)	11±5	10±6	10±5	12±5	12±5	10±6
Sex (males/females)	158/76 (10 N/A)	41/21 (3 N/A)	37/20 (5 N/A)	18/12 (1 N/A)	34/10 (1 N/A)	28/13
Height (cm)	141±33	136±34	141±51	144±28	145±27	132±31
Weight (kg)	44±37	46±30	38±22	44±21	45±23	40±24
Burn size (% TBSA)	55±17	52±16	52±17	58±15	55±18	53±16
% third burn	41±23	36±21	36±22	47±24	41±22	38±22
DPB	76±106	6±2	23±5	68±15	174±36	354±33

Table 1 Patient demographics for Physiological and Functional Evidence

Data are presented as average ± standard deviation.

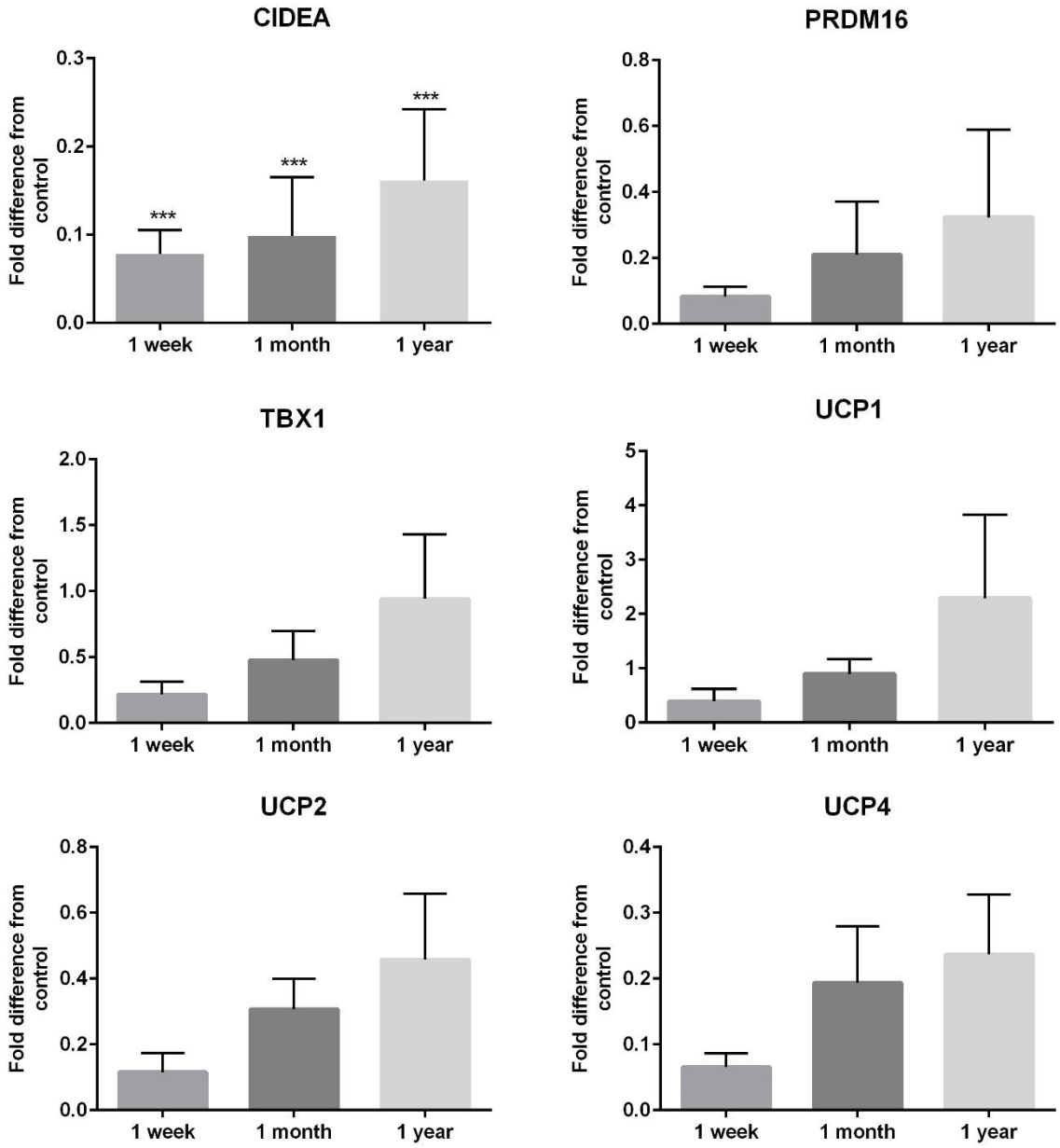
The demographics of the burned patients provided samples which produced the molecular evidence of browning (see "Molecular Evidence"), are presented in Table 2.

	1 week (n=4)	**1 month (n=6)**	**1 year (n=3)**
Age (years)	15±2	14±2	16±2
Sex (males/females)	2/2	4/2	3/0
Height (cm)	166±7	167±6	170±6
Weight (kg)	67±7	60±12	69±18
Burn size (% TBSA)	69±8	62±15	53±17
% third burn	56±20	49±22	33±13
DPB	5±3	28±20	364±401

Table 2 Patient demographics for Molecular Evidence

Data are presented as average ± standard deviation.

Molecular Evidence of Browning



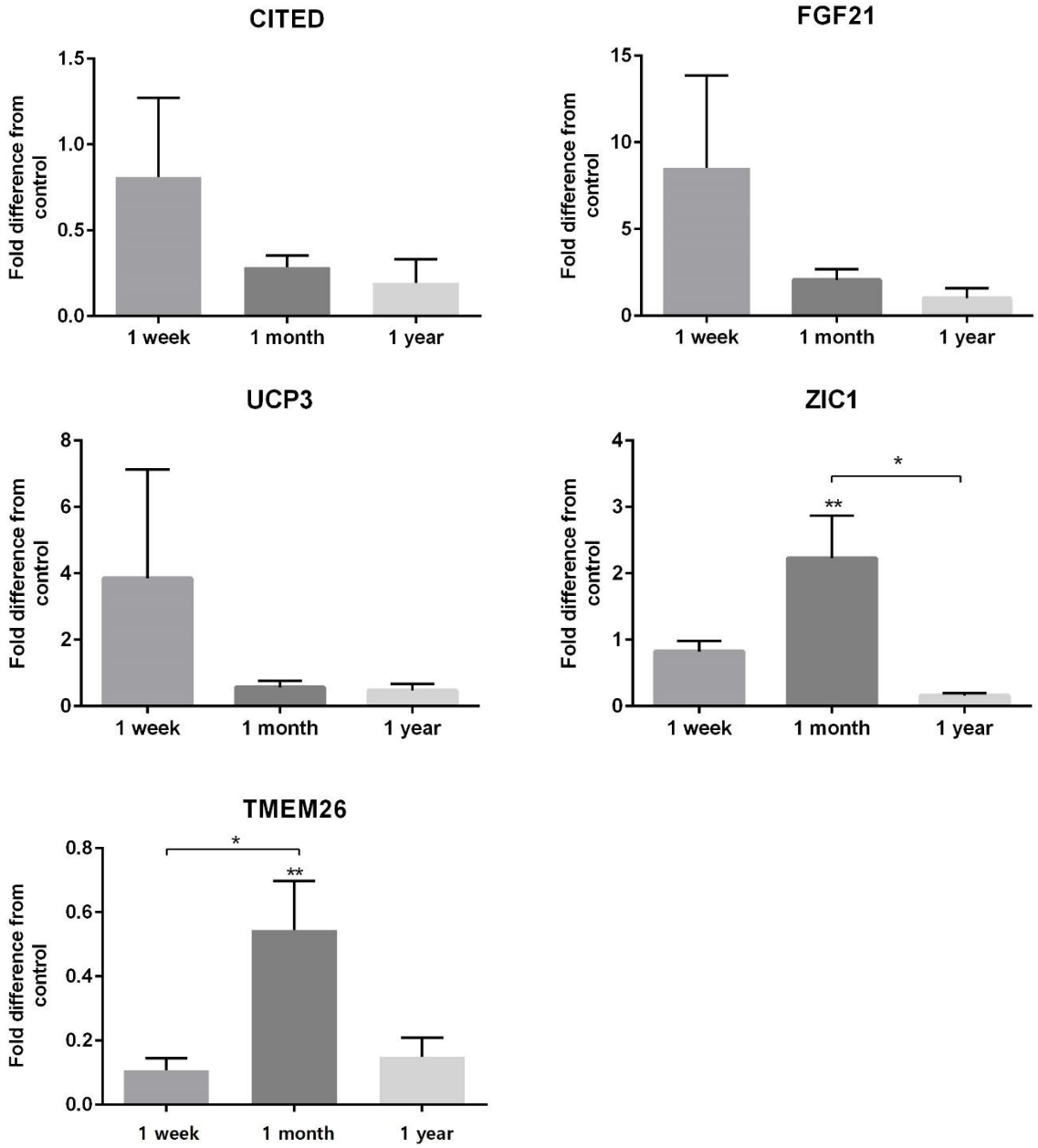


Figure 6: Molecular evidence of browning

The expression of specific genetic browning markers is presented as fold difference of burned patients from healthy individuals.

Values are presented as group means ± SE.

*= p≤0.05, **= p≤0.01, ***= p≤0.0001

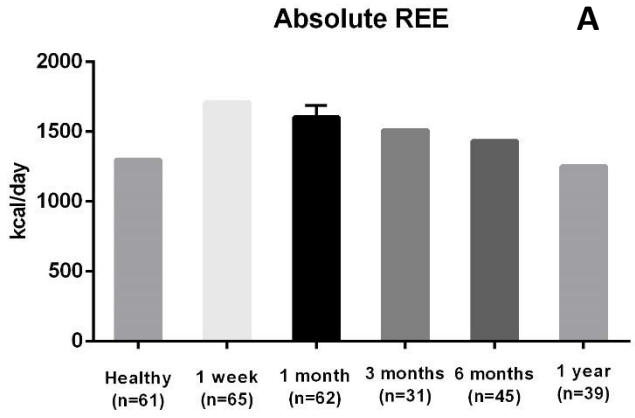
Real-time PCR revealed changes in the expression of specific markers of beige and brown adipocytes from the day of burn until 1 year after injury. Some markers (CIDEA, PRDM16, TBX1, UCP1, UCP2, UCP4) increased gradually during the year followed the burn injury, others decreased during the same time (CITED, FGF21, UCP3) and the rest fluctuated (ZIC1 and

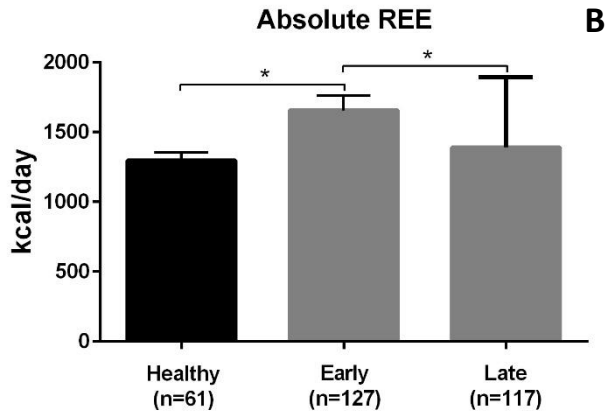
TMEM26). One-way ANOVA produced the following results. CIDEA expression increased significantly (***$p \leq 0.05$) even from the first week and kept increasing even 1 year after burn. PRDM16, TBX1, UCP1, UCP2 and UCP4 followed the same tendency post-burn even though the fold-increase from the healthy controls was not found to be significant.

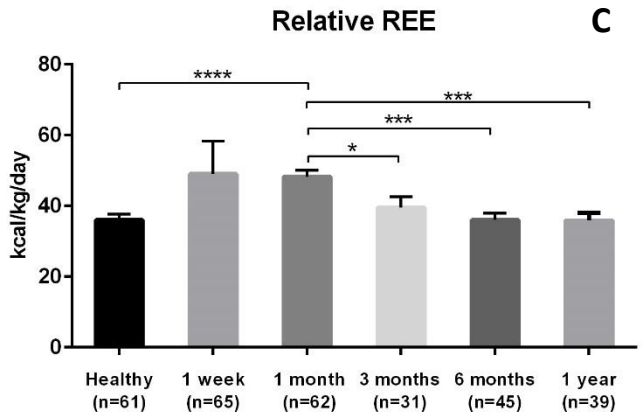
The expression of CITED, FGF21 and UCP3 during the first week after burn, reached a ~0.75, ~8.0 and ~4.0 fold difference from the healthy controls, respectively for each marker. Then, their expression decreased gradually but not significantly, while remained augmented even 1 year after the burn injury.

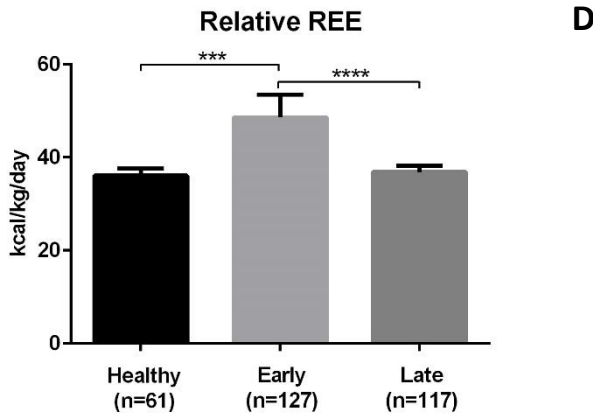
The levels of the brown-specific marker ZIC1 and the beige-specific marker TME26 fluctuated the year following the burn injury. ZIC1 had ~0.9 fold difference from the healthy controls in the 1 week group and a 2-fold difference 1 month after burn. After that, ZIC1 levels decreased reaching significantly lower levels in the 1 year group comparing with the 1 month group. Similarly, TMEM26 increased slightly the 1 week (~0.1 fold-difference) and then increased significantly reaching approximately a 0.6 fold-difference from healthy 1 month after burn.

Physiological Evidence






Relative REE
C
kcal/kg/day
80
60
40
20
0
****
***
***
*
Healthy (n=61)
1 week (n=65)
1 month (n=62)
3 months (n=31)
6 months (n=45)
1 year (n=39)


Relative REE
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kcal/kg/day
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0
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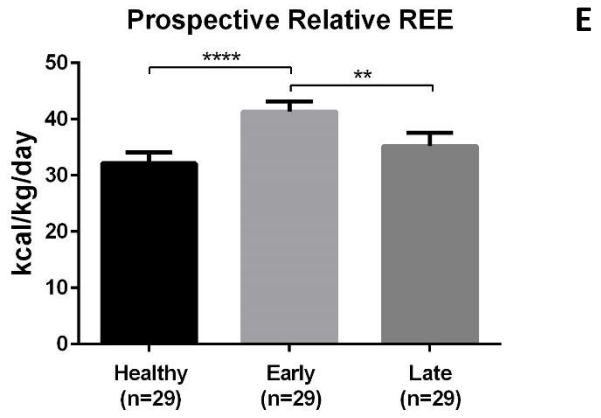
Healthy (n=61)
Early (n=127)
Late (n=117)


Prospective Relative REE
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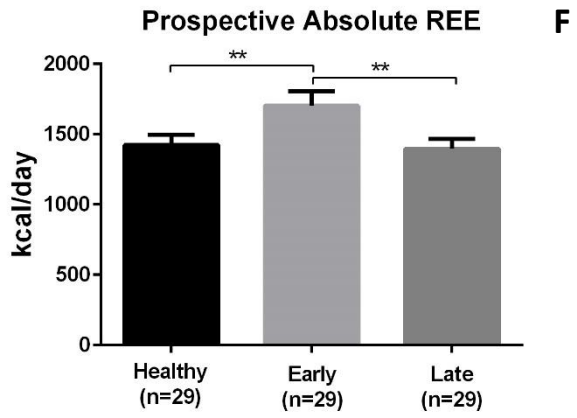
Healthy (n=29)
Early (n=29)
Late (n=29)


Prospective Absolute REE
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kcal/day
2000
1500
1000
500
0
**
**
Healthy (n=29)
Early (n=29)
Late (n=29)

Figure 7

A) Absolute REE increased during the first week after burn and then decreased gradually the following year, with no significant differences between the time groups.

B) The data used were the same as in Figure 7A, while participants were divided in two time groups instead of five, which are the Early (1-45 DPB) and Late (46-400 DPB) group, and were compared with healthy values. Early group had significantly higher values of absolute REE relative to controls ($p \leq 0.05$) and Late group had significantly lower absolute REE in comparison with the Early group ($p \leq 0.05$).

C) Relative REE calculated by subtracting body weight on the day of measurement, from absolute REE. In general, relative REE increased significantly the month following the burn injury and then decreased significantly, almost reaching similar levels of REE as the healthy group.

D) The data used were the same as in Figure 7C, while participants were divided in two time groups instead of five, the Early (1-45 DPB) and Late (46-400 DPB) group, and were compared with healthy values. Relative REE was significantly higher in the Early group compared with the healthy controls, while Late group had significantly lower values compared with the Early group.

E and F) 29 burn patients were followed prospectively and measurements were grouped based on the DPB for each measurement. Measurements in the Early group were taken between 1-45 DPB and those in Late group between 46-400 DPB. Both absolute and relative REE increased significantly early post-burn relative to healthy values ($p \leq 0.01$ for absolute REE and $p \leq 0.0001$ for relative REE) and then decreased significantly in the Late group ($p \leq 0.01$ for absolute and relative REE).

Values are presented as group means ± SE.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Regarding the physiological changes which happen after the burn injury, Figure 7A shows that absolute REE increased during the first week after burn relative to healthy values and then decreased gradually the following year, with no significant differences between the groups. When the participants divided in two time groups, Early (1-45 DPB) and Late (46-400 DPB), the differences between the groups become significant (Figure 7B). More specifically, patients in the early group found to have significantly higher values of absolute REE relative to controls ($p \leq 0.05$), while the late group had significantly decreased levels of absolute REE when compared to the early group ($p \leq 0.05$).

Relative REE was calculated by subtracting body weight on the day of measurement, from absolute REE. Likewise, the values of relative REE didn't pass the normality test and Kruskal – Wallis test was used. In general, Figure 7C shows that relative REE increased significantly the month following the burn injury and then decreased significantly, almost reaching healthy levels of REE. More specifically, analysis showed significant differences between the groups of healthy versus 1 month ($p \leq 0.0001$), 1 month versus 3 months ($p \leq 0.05$), 1 month versus 6 months ($p \leq 0.0001$), and 1 month versus 1 year ($p \leq 0.001$) (Figure 7C). The same results were found with the categorization of participants into two groups (early group 1-45 DPB and late group 46-400 DPB) (Figure 7D).

Several patients (n=29) were followed prospectively for 12 months after the burn injury and measurements were grouped based on the number of DPB for each measurement date. Measurements in the Early group were taken between 1-45 DPB and those in Late group between 46-400 DPB. The results, once again, showed that in the patients followed prospectively, absolute and relative REE increased significantly in the early group relative to healthy values ($p \leq 0.01$ for absolute REE and $p \leq 0.0001$ for relative REE) and then decreased significantly in the late group ($p \leq 0.01$ for absolute and relative REE) (Figures 7E,F).

Functional Evidence

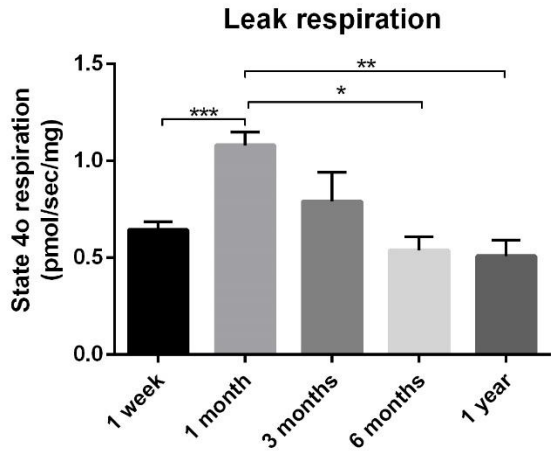

Figure 8

1 week (n=98), 1 month (n=68), 3 months (n=15), 6 months (n=7), 1 year (n=13).

Values are presented as group means ± SE.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Oligomycin-insensitive leak respiration (i.e., state 4^O respiration) was determined in digitonin permeabilized WAT samples following the titration of substrates, ADP and oligomycin. In cohort of severely burned children measured at five time-points after the burn injury (1 week, 1 month, 3 months, 6 months and 1 year) it was observed that mitochondrial activity increased significantly 1 month post-burn compared with the 1 week measurements ($p \leq 0.001$). Then, leak respiration decreased gradually reaching lower levels 1 year post-injury compared to the 1 week group. More specifically, mitochondrial activity found to be significantly lower than the 1 month time-point, first at the 6 month time-point ($p \leq 0.05$) and then at the 1 year time-point ($p \leq 0.01$).

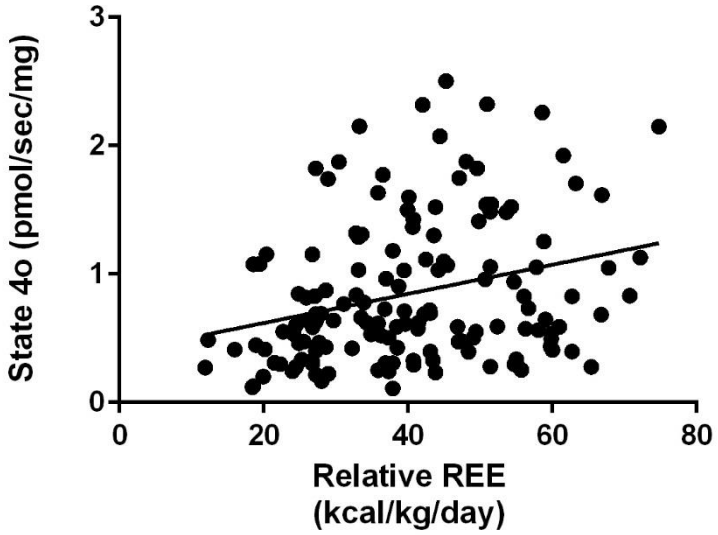

Figure 9 Linear regression of relative REE and state 4o

The increase of leak respiration (state 4o) was proportional to the increase of relative REE during the year followed the burn injury. The graph presented in Figure 9 is described by the following equation: Y=0.01134*X+0.3922. These results suggest that the augmented mitochondrial activity due to browning is positively correlated with the increase of REE after the burn injury.

6. Discussion

The presented work confirms the plasticity of human subcutaneous WAT depots which includes the adoption of a brown-like phenotype after burn injury and recurrence to the white phenotype when the stimulant -adrenergic stress in the case of burn injury- seize to exist. Additionally, it seems that the whitening process starts approximately 1 month after the burn injury. Although, the results of the browning process remain evident even 1 year after burn.

Gene analysis confirmed the browning of WAT in burned children, by measuring specific beige and brown adipose tissue markers. Some markers (CIDEA, PRDM16, TBX1, UCP1, UCP2, UCP4) increased gradually during the year followed the burn injury, others decreased during the same time (CITED, FGF21, UCP3) and the rest fluctuated (ZIC1 and TMEM26). The results of the gene expression in the early samples (1 week, 1 month) agree with previous published findings from the same lab. (L. S. Sidossis et al., 2015)

Resting energy expenditure was measured soon after the burn injury and follow up measurements were obtained during the following year. The measurements showed that REE increased significantly the first week post-burn and then decreased gradually to meet the healthy estimated REE levels. The same tendency observed with those patients who were followed prospectively up to one year after burn. The observation that REE returns to almost healthy levels 1 year post-burn does not agree completely with previous findings. Other studies reported that hypermetabolism endures 1 to 2 years post-burn, but in they reached this conclusion by studying adults or younger children or measuring muscle biopsies instead of sWAT or included patients with 40% TBSA instead of 30% TBSA.(Hart et al., 2000; Jeschke et al., 2011; C. Porter et al., 2014; Craig Porter et al., 2016)

Leak respiration measurements of sWAT samples showed a significant increase of mitochondrial activity 1 month post-burn, followed by a gradual decrease up to 1 year post-burn. It was also observed that leak respiration was positively correlated with REE, suggesting that the increase of mitochondrial activity is at least partially responsible for the hypermetabolism taking place after burn.

With this work, it was confirmed that adrenergic stress caused by burn injury in human children, leads to browning of WAT. Going one step further, proof of the occurrence and time of whitening process was collected. As such, it seems that WAT adopts a more brown-like phenotype in children who have a burn injury to increase the chances of surviving, probably by increasing the heat production while heat eludes body because of the damaged skin tissue which plays distinct role in thermoregulation process. Although, when healing process starts and the burn injury decreases, the activated BAT and/or the differentiated beige adipocytes return to the pre-burn state

leading to decreased expression of brown and beige gene markers, lower levels of REE and reduced levels of mitochondrial activity.

One of the study limitations was the small number of patients used for the gene analysis. Also, a useful addition to those results would have been the morphological and immunohistochemical examination of the WAT samples taken from the patients.

Understanding the mechanisms controlling the browning and whitening processes is of major importance, as they can be the foundations for designing new techniques useful in clinical care. For example, the possibility of controlled browning of WAT in order to improve energy expenditure and insulin sensitivity, would benefit diabetic and obese patients. Also, promoting the whitening process in burned patients may decrease recover time and maybe increase survival rates.

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8. Appendix

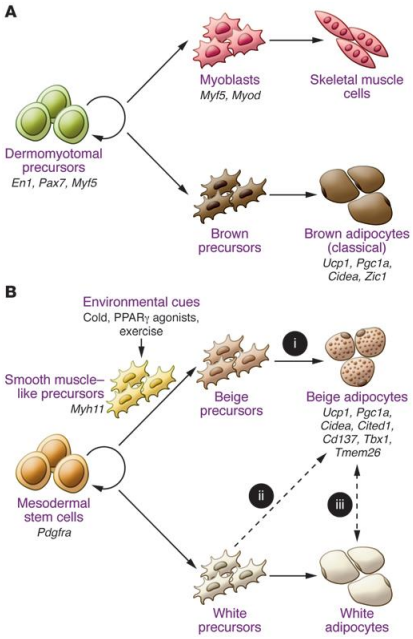

Figure 1 Developmental origins of brown and beige adipocytes (P. Lee et al., 2013)

A) Classical brown adipocytes share the same origins with skeletal muscle cells and can be identified by several markers, including UCP1, PGC1a, CIDEA and *ZIC1*.
B) A subgroup of beige adipocytes originates from $MYH11^{+}$ smooth muscle-like precursors. Differentiation is regulated by environmental factors, such as cold exposure, PPARγ agonists and exercise. Beige adipocytes express several markers, including UCP1, PGC1a, CIDEA, CD137, TBX1, TMEM26 and CITED1. Beige adipocytes may be developed from (i) distinct beige precursors, (ii) direct differentiation of white precursors, or (iii) transdifferentiation from mature white adipocytes.

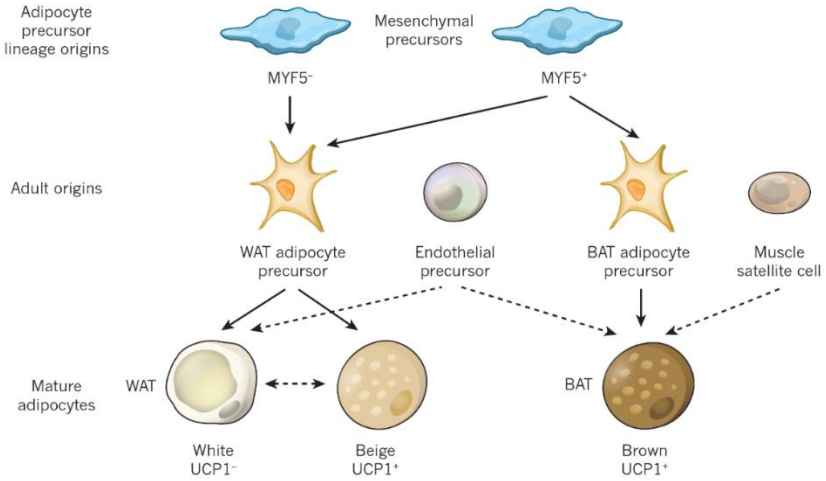

Figure 2 Origins of white, beige and brown adipocytes. (Peirce, Carobbio, & Vidal-Puig, 2014)

Classic brown adipocytes express UCP1, while WAT contains both white (UCP1-) and beige (UCP1+) adipocytes. BAT adipocyte precursors derive from mesenchymal precursor cells expressing MYF5, while WAT precursors develop from both MYF5- and MYF5+. Beige adipocytes can derive from WAT adipocyte precursors and possibly directly from mature white adipocytes.

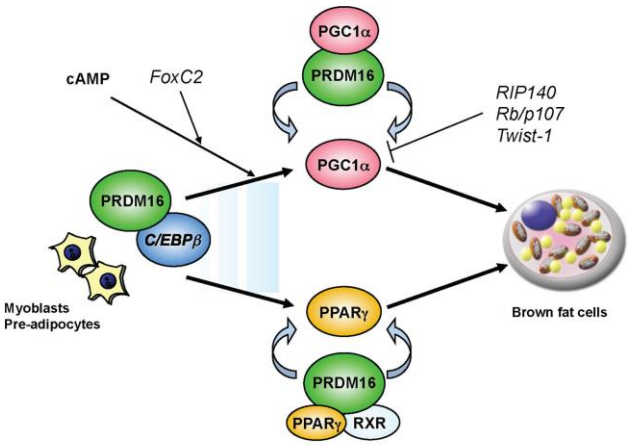

Figure 3 Transcriptional control of brown fat development through PRDM16 (Kajimura, Seale, & Spiegelman, 2010)

The complex of PRDM16 with C/EBP-β acts in MYF5+ myoblastic precursors or pre-adipocytes to induce expression of PPARγ and PGC-1α. PRDM16 co-activates PPARγ and PGC-1α, which leads to brown fat differentiation.

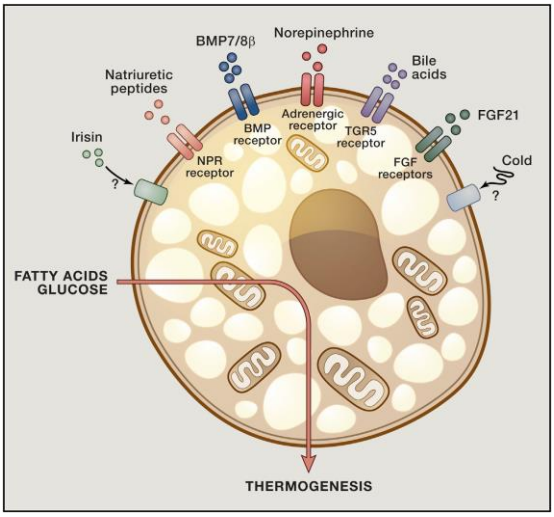

Figure 4 Activators of Beige and Brown adipocytes development and function (Rosen et al., 2014)

Browning and increased thermogenesis are induced by several factors. Some of these factors appear to induce the development of new beige adipocytes, like irisin, or brown adipocytes, like BMP7, while others may affect both recruitment and enhancement of thermogenic activity.

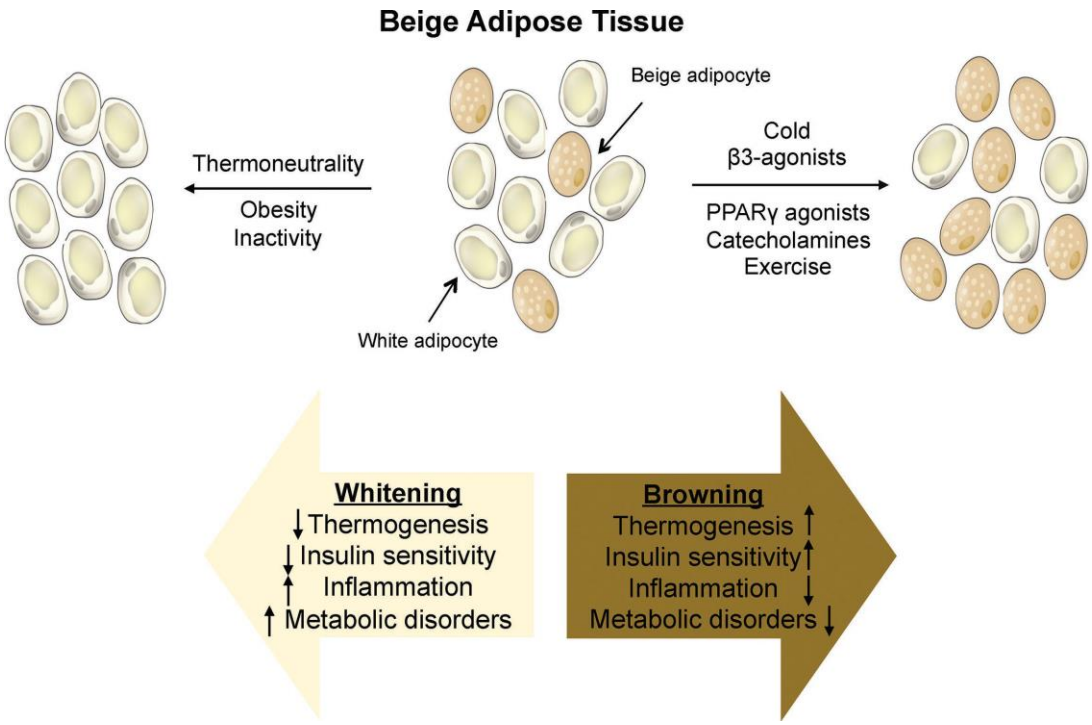

Figure 5 Representation of whitening and browning (Cohen & Spiegelman, 2016)

Representation of browning, which is characterized by the development of beige adipocytes among white adipocytes. Browning is characterized by increased thermogenesis and insulin sensitivity, while having decreased inflammation. Whitening is the reverse process, thus characterized by the opposite conditions.