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Revealing the importance of solute carrier transporters in thermogenic activity of human adipocytes

by

Rini Arianti

Supervisor: Prof. Dr. László Fésüs

UNIVERSITY OF DEBRECEN

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By **Rini Arianti (M. Sc)**

Supervisor: **Prof. Dr. László Fésüs**

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the Defense Committee : Prof. Dr. Gábor Szabó, DS. C.

Reviewers:

1. Dr. Beáta Lontay, Ph.D.
2. Dr. Timea Komlódi, Ph.D.

Members of the Defense Committee:

1. Dr. Róza Zákány, Ph.D.
2. Dr. Csilla Laczka, Ph.D.

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INTRODUCTION

World Health Organization reported in 2016 that more than 1.9 billion adults aged 18 years and older were overweight and of these over 650 million adults were obese [World Health Organization, 2021]. In the last decade, a novel interest has emerged to explore pathways that regulate energy homeostasis in mammalian system, particularly focusing on adipose tissues. There are two types of adipose tissue: white adipose tissue (WAT) that functions as lipid storage and brown adipose tissue (BAT) that function in thermogenesis. White adipocytes possessed unilocular lipid droplet and low amounts of mitochondria, whereas brown adipocyte have multilocular lipid droplet and high amounts of mitochondria [Cohen and Kajimura, 2021]. White adipocytes residing in WAT also have thermogenic potential which is referred as beige adipocytes. Beige adipocytes emerge when there are stimuli such as cold and exercise and they have thermogenic brown adipocyte-like phenotype [Cohen and Kajimura, 2021].

BAT has been widely studied in recent years as the potential target of obesity therapy. Brown/beige adipocytes have a special feature, uncoupling protein-1 (UCP1) which enables them to generate heat instead of ATP by non-shivering thermogenesis [Cannon and Nedergaard, 2004]. Glucose and fatty acids are main substrate for thermogenesis suggesting the important role of BAT in systemic glucose and lipid homeostasis. Recent study also reported that branched-chain amino acids (BCAAs) are oxidized by brown adipocytes during thermogenic activation by cold [Yoneshiro *et al*., 2019, *Nature*].

Solute carrier (SLC) transporters family members are involved in various biological processes, mainly in transporting the important molecules needed by cells. The importance of cell membrane SLCs in thermogenic activation of human brown/beige adipocytes have not been elucidated. Here we revealed the importance of amino acids and thiamine transporters in thermogenic activation of human adipocytes upon adrenergic stimulation. The inhibition of these transporters during cAMP stimulation led to the decrease of heat generation reflected by proton leak respiration. In addition, cAMP-induced upregulation of thermogenic markers were also abrogated when the activity of amino acid and thiamine transporters are blocked by their pharmacological inhibitors. Our results have raised the possibility to treat obesity by targeting the molecular elements of the regulation of amino acids and thiamine metabolism to augment heat generation by thermogenic adipocytes in obese individuals.

Distribution of human brown/beige adipose tissue

Human BAT was primarily regarded as a tissue that was only present in infants and located at anatomical sites that are difficult to reach. Several studies using positron emission tomography (PET) provided evidence that adults have significant amounts of BAT. The combination of labeled glucose uptake and PET has proven that cold exposure can activate the BAT. The most common location for brown adipose tissue in adult humans is the cervical-supraclavicular depot marked by high labeled glucose uptake in that region [Cypess *et al*., 2009; Virtanen *et al*., 2009]. Assessing the morphology of the biopsies specimen from supraclavicular depot, they found adipocytes with numerous multilocular and intracellular lipid droplets.

A more recent study had improved the PET-CT method to identify the brown/beige adipose tissue localization [Leitner *et al*., 2017]. Using a refined technique of PET-CT method, they could identify the whole-body BAT distribution and estimate its thermogenic capacity. They found that BAT and brownable adipose tissue can be found interspersed in several areas such as cervical, supraclavicular, axillary, mediastinal, paraspinal, and abdominal. Intriguingly, they also found that obese individuals possess higher amount of brownable adipose tissue than lean individuals. However, the active BAT in obese people is lower than in lean individuals.

Heat generation in brown/beige adipose tissue: UCP1-dependent and UCP1-independent thermogenesis

The thermogenic capacity of brown/beige adipocytes depends on fatty acids oxidation through oxidative metabolism [Gonzalez-Hurtado *et al*., 2018]. Thermogenesis is classically activated by adrenergic signaling mediated by sympathetic neuron system, which enhances lipolysis through cAMP-PKA signaling pathway. Long-chain fatty acids released from lipolysis serve as metabolic fuel and at the same time also allosterically activate the UCP1 function [Sakers *et al*., 2022]. UCP1 catalyzes the proton leak in mitochondrial inner membrane enabling the brown/beige adipocyte to expend energy as heat instead of ATP. The accumulated protons in the intermembrane space during respiratory chain can be brought back into the mitochondrial matrix by long-chain fatty acid that bound to UCP1.

UCP1 has been the center player of non-shivering thermogenesis. The disruption of function or the deletion of UCP1 in mice result in more sensitivity to acute cold exposure but not obese [Enerback *et al*., 1997]. However, when the temperature is gradually decreased to 4^{o}C, UCP1 KO mice are still able to adapt to cold indicating that there is a compensatory mechanism in the lack of UCP1 [Ukropec *et al.*, 2006]. Cold-acclimated UCP1 KO mice expressed higher level of brown markers in inguinal WAT with more beige adipocytes as compared to wild-type indicating that more beiging occurs to compensate the UCP1 depletion. UCP1-deficient WAT exhibits elevated expression of mitochondrial creatine kinase (*CKMT*), *DIO2*, calcium-ATPase, *PGC1a*, and *COX2* [Kazak *et al*., 2015]. The mechanisms of UCP1-independent thermogenesis involve creatine futile cycle, ca^{2+} futile cycle, lipidated amino acids, and triacylglycerol futile cycle [Chouchani and Kajimura, 2019; Ikeda *et al*., 2017; Kazak *et al*., 2015; Long *et al*., 2016]. UCP1-independent thermogenesis requires glycolysis and mitochondrial ATP synthesis to supply fuel for futile cycling [Sakers *et al*., 2022].

The creatine-driven futile substrate cycle can augment energy expenditure by releasing high-energy phosphate from phosphorylated creatine [Kazak *et al*., 2015]. Phosphocreatine is the main energy source in skeletal muscle during high intensity exercise to support the muscle contraction. The phosphocreatine/creatine cycle can generate ATP by directly phosphorylating ADP. In brown/beige adipocytes, creatine metabolism-related genes such as creatine transporter (*SLC6A8*), guanidinoacetate methyltransferase (*GAMT*), and glycine amidinotransferase (*GATM*) increase during cold exposure [Kazak *et al*., 2017]. Recent study reported that creatine kinase B (*CKB*), localized in mitochondria, play a main role in phosphorylating creatine in brown/beige adipocytes

[Rahbani *et al*., 2021]. The expression of *CKB* is the highest in the human abdominal SC-derived adipocytes as compared to other creatine kinase isoform and forskolin increase its expression further [Rahbani *et al*., 2021]. Tissue non-specific alkaline phosphatase (*TNAP*), also localized in mitochondria of adipocytes, serves as phosphocreatine phosphatase in the creatine futile cycle that occurs in the adipocyte mitochondria [Sun *et al*., 2021]. The expression of both *CKB* and *TNAP* elevate in brown/beige adipocyte during cold exposure.

Thermogenic genes expression in human neck tissue

Study in human adipose tissue isolated from neck area showed a higher expression of UCP1, LHX8, and ZIC1 in DN as compared to SC adipose tissue [Cypess *et al*., 2013]. The mRNA expression of those classical BAT markers was also enriched in primary adipocytes derived from human fetal interscapular adipose tissue [Seiler *et al*., 2015]. However, another study reported that the mRNA expression of ZIC1 negatively correlated to mature brown adipocytes activity such as oxygen consumption rate [Nascimento *et al*., 2017].

Shinoda *et al*. (2015) performed RNA-sequencing analysis to identify molecular markers of clonally derived human brown adipocytes isolated from supraclavicular. They reported that the population of UCP1-positive cells displayed beige adipocyte characteristic. Moreover, they revealed that microtubule-associated tumor suppressor 1 (*MTUS1*) and potassium channel subfamily K member 3 (*KNCK3*) were essential for beige adipocyte differentiation and thermogenic activity. Another study investigated the identity of the brown adipose tissue biopsies and found that BAT biopsies displayed the beige characteristic, which is marked by higher expression of beige markers including *CD137*, *TMEM26*, and *TBX1* [Wu *et al*., 2012]. The gene markers of classical brown adipose tissue such as *EBF3*, *EVA1*, and *FBXO31* were expressed at the same level between BAT and white adipose tissue (WAT). Another study revealing the resemblance between human BAT and mice beige adipocytes was reported by Sharp *et al*. (2012). They analyzed the mRNA expression of brown and beige markers in total RNA isolated from various anatomical locations, including subcutaneous supraclavicular areas, posterior mediastinum, retroperitoneal, intra-abdominal, mesenteric depots, and thigh tissues. Total RNA isolated from WAT and smooth muscle were used as controls. Beige-selective markers such as *HOXC8*, *HOXC9*, and *CITED1* were highly expressed in human BAT, whereas classical brown adipose tissue markers were not detectable. These studies suggested that human BAT, isolated from various anatomical locations, are more resemble to mice beige adipocytes.

Computational tools had been developed to explore the characteristic and thermogenic capacity of brown/beige adipocytes samples. These web tools, which is publicly available online, were developed based on the in-depth analysis of large transcriptomic data of mice white, beige, and brown adipocytes, in case of BATLAS both mice and human adipocytes transcriptomic data were utilized. BATLAS and ProFAT web tool were developed to estimate brown adipocyte content and browning probability of heterogeneous population of adipocytes [Perdikari *et al*., 2018; Cheng *et al*., 2018].

SLC transporters: classifications and functions

Solute carrier (SLC) transporters participate in various biological processes by transporting essential nutrients, both macro and micronutrients, disposing the waste products, and shuttling the metabolites between organels [Zhang *et al*., 2019; Kenthirapalan *et al*., 2016]. SLC transporters serve as metabolic gates and enable the cells to uptake the available substrates to generate energy or to maintain homeostasis. Other physiological function including tissue development, oxidative stress, and neurological regulation also require the SLC transporters contribution. Cell membrane transporters majorly include members of ion and water channels, consisting of ATP-binding cassettes (ABC) and SLC transporters [Zhang *et al*., 2019]. Human SLC transporter families consist of 400 genes and 52 subfamilies. A comprehensive similarity relationship of SLCs superfamily is described in. SLCs play important role in transmembrane transport of various substrates such as inorganic ions, fatty acids, amino acids, and neurotransmitters.

There are 4 modes of transmembrane transport by SLCs: cotransporter/coupled transporter, exchanger, facilitated/passive transporter, and orphan transporter. Cotransporter is defined as the transport of one substrate via concentration gradient coupled with other molecules [Zhang *et al*., 2019]. Sodium/glucose co-transporter, which belongs to *SLC5A* family, was the first discovered cotransporter. It transports Na^+ and glucose in two directions across the epithelial cell membrane providing glucose for glycolysis. Exchanger mode transports two substrates across the membrane in the opposite direction. Sodium/hydrogen exchanger 3, encoded by *SLC9A3*, facilitates the transport of Na^+ and H^+ in opposing direction to eliminate acids generated by active metabolism thus pH in the gut is maintained at normal condition [Zhang *et al*., 2019; Hediger *et al*., 2012]. The third mode of transmembrane transport is the facilitated transporter that mediate the substrate translocation without coupled molecules or by spontaneous passive transport. Thiamine transporter 2, encoded by *SLC19A3* and later will be described more detail, is one of the facilitating transporters. A major player in thermogenesis UCP1, also known as *SLC25A7*, transfers the H^+ from inner to outer mitochondrial membrane via facilitated transport. The orphan transporter comprised the transporters with unidentified substrates and function [Hediger *et al*., 2012].

Alanine-serine-cysteine transporter-1

Human ASC-1 is the light subunit encoded by *SLC7A10* and belongs to the LAT subfamily and is bridged through Cys154 to 4F2hc heavy subunit [Yan *et al*., 2019]. ASC-1 is a sodium-independent transporter that has high affinity for the small neutral D- and L-amino acids, preferentially by exchanger mechanism. It has a main role in modulating the glutamatergic transmission through mobilization of D-serine at the glutamatergic synapse [Brown *et al*., 2013]. ASC-1 has multiple substrates such as L-alanine, L-cysteine, glycine, and both isoform of serine. ASC-1 is abundantly expressed in brain and is being investigated as a therapeutic target for schizophrenia [Rosenberg *et al*., 2013]. Inhibition of ASC-1 may elevate the level of extracellular glycine and D-serine in critical brain regions. A small molecule BMS-466442 has been characterized as a selective inhibitor of ASC-1 with IC_{50} 36.8±11.68 nM and 19.7±6.7 nM for human ASC-1 expressing cells and primary cultures [Brown *et al*, 2013].

The role of ASC-1 in synaptic system has been elucidated, however, its role in metabolic regulation has not been explored. ASC-1 was identified as surface marker of mice white adipocytes [Ussar *et al*., 2014]. The protein expression of ASC-1 is higher in cell membrane of SC WAT as compared to BAT (Ussar *et al*., 2014). Later study reported that a subpopulation of preadipocytes expressing ASC-1 exerted lower capacity to differentiate to beige adipocytes as compared to ASC-1 negative cells [Suwandhi *et al*., 2021]. Other study reported that ASC-1 expression is negatively correlated with waist-to-hip ration and its impairment by BMS-466442 decreased OCR of human primary adipocytes [Jersin *et al*., 2021]. The inhibition of ASC-1 in adipocytes also reduced serine uptake and total glutathione levels. As the consequences, ROS production was promoted leading to oxidative stress and cellular damage [Jersin *et al*., 2021].

Thiamine transport and metabolism

Free thiamine uptake is performed by thiamine transporter 1 (ThTr1) and 2 (ThTr2), which belong to *SLC19* family alongside with folate transporter (encoded by *SLC19A1*). Both thiamine transporters are located in cell membrane with 12 transmembrane domains. The expression of both transporters is enriched in small and large intestines. In human, ThTr1 is expressed in the apical and basolateral membrane domains of polarized enterocytes and ThTr2 is expressed in the apical brush-border membrane domain (Said *et al*., 2004]. Human ThTr2 seems to play more significant role in thiamine uptake in intestine because the deficiency of ThTr1 does not affect the plasma thiamine levels. Both thiamine transporters mediate the uptake of thiamine, which exists as cation at physiological pH, across the cell membrane using differences in pH as a driving force [Ganapathy *et al*., 2004]. Thiamine influx into cells via ThTrs is enhanced by an outwardly directed H+ gradient ($pH_{out}>pH_{in}$), suggesting thiamine/H+ antiport as the transport mechanism.

Thiamine is present in humans in various forms such as free-thiamine, thiamine monophosphate (TMP), TPP, and thiamine triphosphate (TTP) [Losa *et al*., 2005]. Functional activity of the tissues determines the form of presence thiamine. The largest amount of thiamine exists as TPP, counting for 80% of total body thiamine. TPP is essential as it participates in various biological processes in the cytosol, mitochondria, and peroxisome. In the cytosol, TPP plays role in the pentose phosphate pathway, involved as a cofactor of transketolase enzyme. Transketolase is an important linker of pentose phosphate pathway to glycolysis, feeding excess sugar phosphate into the main carbohydrate metabolic pathways. The pentose pathway plays main role in producing the reducing equivalents, NADPH, that are important for ribose-5-phosphate generation for high-energy ribonucleotide synthesis. The status of low thiamine level can be reflected by abnormal expression and activity of transketolase enzyme [Ortigoza-Escobar *et al*., 2016].

In the mitochondria, TPP is an important cofactor for several complexes including pyruvate dehydrogenase complex (catalyzing the conversion of pyruvate into acetyl-CoA), oxoglutarate dehydrogenase complex (catalyzing the carboxylation of α-glutarate in tricarboxylic acid (TCA) cycle), and branched-chain α-ketoacid dehydrogenase (BCKDH) complex (catalyzing the decarboxylation of branched short-chain α-ketoacids) [Ortigoza-Escobar *et al*., 2017; Tittman, 2009]. TPP is the coenzyme for E1 component of BCKDH complex, leading to its activation. In

contrast, BCKDH kinase (BDK) phosphorylates E1 of BCKDH leading to its inactivation. It has been reported that BDK is also sensitive to the inhibition by TPP [Harper *et al*., 1984]. The TPP-binding site of the E1 component is associated with the active site of the BCKDH, which is also the phosphorylation region of BDK. Noguchi *et al*. (2018) reported that TPP inhibited BDK in BCKDH-BDK complex and the mechanism of inhibition was significantly increased by the presence of free Ca^{2+} in a physiological range concentration. Their finding explains the mechanism of the elevation of branched-chain amino acid (BCAA) oxidation during skeletal muscle exercise by the increase of mitochondrial free Ca^{2+} level. Yoneshiro *et al*. (2019) reported that human brown adipose tissue regulates the circulating BCAA levels upon cold exposure through mitochondrial BCAA transporter encoded by SLC25A44 gene. The clearance of circulating BCAA by BAT resulted in the elevation of BCAA oxidation by BCKDH and controlled the energy homeostasis. The presence of thiamine during BCAA clearance by BAT is essential to maintain the activity of BCKDH.

AIM OF THE STUDY

1. To find differentially expressed genes (DEGs) in human subcutaneous neck (SC) and deep neck (DN) derived white and brown adipocytes.

    - Characterizing the molecular identity of human brown adipocytes derived from deep neck region.
    - Predicting the brown adipocyte content and browning probability of human neck derived adipocytes by using available web tools.

2. To reveal the importance of alanine-serine-cysteine transporter-1 (ASC-1) and thiamine transporters (ThTrs) on thermogenesis of human neck-derived adipocytes.

    - Studying the effect of potent inhibitors of ASC-1 and ThTrs on heat generation of human neck-derived adipocytes by monitoring oxygen consumption and quantification of thermogenesis by determining uncoupling activity of UCP1.
    - Elucidating the important role of ASC-1 in mediating the uptake of amino acids in deep neck adipocytes by measuring amino acids concentration in the conditioned culture fluid during adrenergic stimulation in the presence of ASC-1 selective inhibitor.
    - Investigating the consequence of the restricted uptake of amino acids or thiamine on the expression of thermogenic genes and mitochondrial complex subunits.
    - Evaluating the thiamine direct stimulation on mitochondrial thiamine pyrophosphate-dependent pyruvate dehydrogenase (PDH) activity in cell membrane-permeabilized adipocytes by measuring mitochondrial and proton leak respiration driven by PDH substrate.
    - Analyzing the effect of ThTrs potent inhibitors on the expression of thermogenic genes in human neck tissue biopsies.

MATERIALS AND METHODS

Isolation of SC and DN derived human adipose stromal cells (hASCs)

During thyroid surgeries, a pair of DN and SC adipose tissue samples was obtained to rule out inter-individual variations. Patients with known diabetes, malignant tumor or with abnormal thyroid hormone levels at the time of surgery were excluded. Adipose tissue specimens were dissected from blood vessels and connective tissues and minced into small pieces. The minced tissues were digested in 120 U/mL-containing PBS for 1 hour in 37ºC water bath. The tube was gently mixed every 15 minutes. The completely disaggregated tissue was filtered to remove the tissue debris. The cell suspension was centrifuged for 10 minutes at 200 g and the pellet of SVF was re-suspended in DMEM-F12 (Sigma Aldrich) medium containing 10% FBS, 100 U/mL penicillin-streptomycin, 33 µM biotin and 17µM pantothenic acid. hASCs were seeded into 6-well plates at a density of 15000 cells/cm^2 and cultured in serum-containing medium at 37ºC in 5% CO^2 for 24 hours to attach. Floating cells were removed with PBS and the attached hASCs were cultures until they reached confluency. The mycoplasma-free was checked by PCR analysis.

Adipocytes differentiation: white and brown adipocytes

Human stromal vascular fraction were isolated and differentiated into adipocytes (ADIP) under regular adipogenic medium and brown adipocytes (B-ADIP) under long-term rosiglitazone treatment. ADIP differentiation was induced for three days using DMEM-F12 medium supplemented with 33 µM biotin, 17 µM pantothenic acid, 10 µg/mL human Apo-transferrin, 20 nM human insulin, 100 nM hydrocortisone, 200 pM T3, 2 µM rosiglitazone, 25 nM dexamethasone, and 500 µM IBMX [Toth *et al*., 2020]. B-ADIP differentiation was induced for three days using DMEM-F12 medium supplemented with 33 µM biotin, 17 µM pantothenic acid, 10 µg/mL apo-transferrin, 0.85 µM human insulin, 200 pM T3, 1 µM dexamethasone, and 500 µM IBMX. After three days, dexamethasone and IBMX were omitted from both differentiation medium. From day 4-14, hydrocortisone was constantly present in the ADIP differentiation cocktail [Kristof *et al*., 2015]. Rosiglitazone and higher concentration of insulin were presence in the B-ADIP differentiation cocktail since day 4-14 of differentiation [Elabd *et al*., 2009; Toth *et al*., 2020; Arianti *et al*., 2021].

Cell culture and pharmacological inhibitor of SLC target treatment

SC and DN preadipocytes were seeded on 6-well plates and differentiated into ADIP and B-ADIPs. After 14 days of differentiation, ADIPs and B-ADIPs were treated with single bolus of dibutyryl-cAMP (Sigma Aldrich) 500 µM for 10 hours. BMS-466442 100 nM was administered to selectively inhibit ASC-1. Fedratinib 1µM (Selleck Chemicals) or amprolium 300 µM (Sigma Aldrich) was administered to inhibit ThTr activity. Oxythiamine 300 µM (Sigma Aldrich) or devimistat 75 µM (MedChem Express) was administered to inhibit the activity of TPP-requiring enzymes. In thiamine concentration dependence thermogenic experiments, we used custom-made thiamine free culture fluid treating adipocytes with the gradually increasing concentration of

thiamine 40 nM, 200 nM, 1 µM, 5 µM, or 25 µM in the presence or absence of cAMP stimulation for 10 hours.

Total RNA isolation and RT-qPCR

Total RNA was isolated from differentiated adipocytes using triazole reagent. Total RNA concentration was measured by nanodrop spectrophotometer after DNase treatment. TaqMan reverse transcription reagent was applied to generate cDNA by following manufacturer's instruction. A LightCycler 480 (Roche Diagnostics) was utilized to determine normalized the expression of gene interest. Validated TaqMan qPCR assay designed and supplied by Applied Biosystems were used according to manufacturer's instruction. Human GAPDH was used as endogenous control. All samples were analyzed in triplicate. Gene expression values were calculated by the comparative Ct method. ΔCt represents the threshold cycle (Ct) of the target minus that of GAPDH.

Western blot

Differentiated ADIPs and B-ADIPS were washed with cold PBS and collected in SDS buffer. The samples were boiled in 100oC for 10 minutes and loaded onto SDS polyacrylamide gel. Proteins were transferred onto PVDF Immobilon-P Transfer Membrane followed by blocking in Tris-buffered saline containing 0.05% Tween-20 TBS (TTBS) and skimmed milk for 1 hour. Membranes were probed by primary antibodies overnight at 4oC. Next, membranes were washed with TTBS 3x15 minutes and followed by incubation with horseradish-peroxidase-conjugated species-corresponding secondary antibodies for 1 h at room temperature. Immunoblots were developed with Immobilon western chemiluminescent substrate. Densitometry analysis of immunoblots was performed by using Image J software.

Oxygen consumption measurement by Seahorse

OCR and ECAR were measured using an XF96 oxymeter (Seahorse Biosciences, North Billerica, MA, USA). Small chamber was created between cells and sensors allowing us to detect a very rapid changes of oxygen concentration as the cells respire to produce ATP through oxidative phosphorylation. XF Seahorse analyzer provides a kinetic measurement of respiration by measuring the concentration of oxygen consumed by cells from the medium (oxygen consumption rate). XF seahorse analyzer also detect the lactic acid production through glycolysis by measuring protons released by cells that acidify the medium (extracellular acidification rate). After recording the baseline OCR, 500 µM dibutyryl-cAMP, 100 nM BMS-466442, 1 µM fedratinib, 300 µM amprolium, 75 µM devimistat, 300 µM oxythiamine or combination of cAMP and SLC inhibitor were injected to the cells. Then, stimulated OCR was recorded every 30 minutes. The adipocytes were treated with the creatine analogue 2 mM β-guanidinopropionic acid (β-GPA) which interferes with creatine-driven substrate cycle [Kazak *et al*., 2015]. Proton leak respiration was determined after injecting ATP synthase blocker (oligomycin) at 2 µM concentration. The respiration remaining after oligomycin injection is linked to the proton leak rate across the inner mitochondrial

membrane [Ruas *et al*., 2016]. Cells received a single bolus of Antimycin A at 10 µM concentration for baseline correction (measuring non-mitochondrial respiration). The OCR was normalized to protein content.

Amino acid consumption measurement

Frozen cell culture supernatants were filtered using 3 kDa filters (Pall Corporation, Port Washington, NY, USA), then 10 µl of filtrate was derivatized with AccQ·Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). Chromatographic separation was carried out on H-class UPLC (Waters) using AccQTag Ultra Column (2.1 X 100mm), AccQTag Eluent A and B and gradient provided in the AccQ-Tag Ultra Chemistry Kit (Waters). Detection of amino acid derivatives was performed at 260 nm in the PDA detector of the UPLC. Concentration of the amino acids was calculated with the Empower software (Waters) using a 7-point calibration curve.

Flux of amino acids into or from adipocytes was calculated by comparing concentration differences measured at starting and end point of 10 hours dibutyryl-cAMP treatment with or without the presence of ASC-1 inhibitor. The number of cells in wells was calculated using KOVA glasstic slide 10 with grids (Kova International Inc, Garden grove, California, USA, cat#K304680) as described in the manufacturer's manual (https://www.kovaintl.com/downloads/DI-91064-17).

Oxygen consumption measurement in cell membrane-permeabilized adipocyte

To study the direct effect of TPP on PDH activity, a Seahorse-based assay was optimized for adipocytes [Mikulas *et al*., 2020]. To SC and DN derived adipocytes a mitochondrial assay solution containing 600 mM mannitol, 210 mM sucrose, 30 mM KH2PO4, 1.5 mM MgCl2, 6mM HEPES, 3 mM EGTA, 0.6% BSA, 4 mM ADP, and 10 mM pyruvate + 5 mM malate, pH 7.4, was added before the measurement. Membrane permeabilizer at 1 nM concentration and 300 µM TPP were administered to the adipocytes after recording the basal OCR for 10 minutes. Then, OCR of permabilized adipocytes was recorded every 5 minutes. Proton leak respiration was determined after injecting oligomycin at 2 µM concentration. Permeabilized cells received a single bolus of Antimycin A at 10 µM concentration for baseline correction (measuring non-mitochondrial respiration).

RNA-sequencing analysis

Total RNA sample quality was checked on Agilent BioAnalyzer using Eukaryotic Total RNA Nano Kit according to the Manufacturer's protocol. Samples with RNA integrity number (RIN) value > 7 were accepted for the library preparation process. Libraries were prepared from total RNA using NEBNext® Ultra™ II RNA Library Prep for Illumina (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads then the mRNAs were eluted and fragmented at 940 °C. First-strand cDNA was generated by random priming reverse transcription and after second strand synthesis step, double-stranded cDNA was generated. After repairing ends, A-tailing and adapter ligation steps, adapter-ligated fragments were amplified in enrichment PCR and finally

sequencing libraries were generated. Sequencing runs were executed on Illumina NextSeq500 instrument using single-end 75 cycles sequencing. After sequencing, the reads were aligned to the GRCh38 reference genome (with EnsEMBL 95 annotation) using STAR aligner (version 2.7.0a). FeatureCounts was used to quantify our reads to genes. Significantly differentially expressed genes (DEGs) were defined based on adjusted p values < 0.05 and log2 fold change threshold > 0.85. Heatmap was generating by using GraphPad 8.0 and interactome map was constructed by using Gephi 9.0 based on interaction from STRING (https://string-db.org/).

Statistical analysis

The results are expressed as mean±SD. Normality of the data was tested by Kolmogorov-Smirnov test. The comparison between two treatments (comparing control versus treated group) was performed by paired t-test or unpaired t-test. The data were visualized and analyzed by using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Molecular signature of human brown/beige adipocytes derived from deep neck tissue

After 14 days differentiation, adipocytes markers such as GLUT-4 (encoded by SLC2A4), PLIN1, ADIPOQ, and LEP were induced after 14 days differentiation and expressed at similar level in both ADIP and B-ADIP irrespective to anatomical origin. Next, we investigated the expression pattern of classical brown and beige adipocytes. The expression of ZIC1 was not induced by differentiation in either SC or DN derived adipocytes. Other classical brown adipocyte markers such as LHX8 and EPSTI1 was expressed higher in DN derived ADIP and B-ADIP and their expression was already high in DN progenitors as compared to SC derived ones. A selective marker for brown/beige preadipocyte EBF2 [Wang *et al*., 2014], which regulate the brown/beige adipocyte identity, was expressed higher in DN as compared to SC preadipocytes and DN derived adipocytes, both ADIP and B-ADIP, maintained high expression of EBF2 after differentiation. DN derived adipocytes also expressed higher level of beige adipocyte marker TBX1 as compared to SC ones. The expression of UCP1 was induced after differentiation and DN derived adipocytes showed higher UCP1 expression as compared to SC adipocytes irrespective to differentiation protocol. DN derived adipocytes also expressed higher level of genes involved in UCP1-independent thermogenesis such as CKMT1a/b and PM20D1 [Kazak *et al*., 2015; Long *et al*., 2016]. In addition to higher expression of brown/beige adipocyte marker, DN derived adipocytes also possessed higher brown adipocyte content (BATLAS, [Perdikari *et al*., 2018]) and browning degree score (ProFAT, [Cheng *et al*., 2018]) as compared to SC derived ones. Our data indicated that adipocytes derived from different anatomical origins possessed distinct molecular signature and DN derived adipocytes possessed classical brown and beige adipocyte identity. DN derived ADIP and B-ADIP had higher thermogenic capacity as compared to SC ones marked by higher expression level of thermogenic markers and both BATLAS and ProFAT scores.

Differential expression of SLC transporters

Among the differentially expressed genes, we found 21 SLC transporters regulated by either the anatomical location (15 upregulated, 3 downregulated in DN versus SC ADIPs) or the applied differentiation protocol (3 upregulated in both SC and DN B-ADIPs). Among 21 SLC transporters, long chain fatty acid transporter encoded by SLC27A3 is the only transporter localized in mitochondrial membrane, whereas other 20 SLC transporters are cell membrane transporters. SLC transporters are involved in various biological processes mediating the uptake of carbohydrate (SLC45A3, SLC2A12, and SLC37A2), amino acid (SLC7A10 and SLC1A7), fatty acid (SLC27A3), cations/anions (SLC22A11, SLC22A12, and SLCO2B1), vitamins (SLC19A3 and SLC4A1), monocarboxylate (SLC16A4 and SLC16A8), bicarbonate (SLC4A11) and Na+/K+/Ca2+ (SLC24A2 and SLC24A3). The role of these transporters in nutrient uptake in human adipocytes derived from neck tissues have not been explored so far. In the following chapters, the potential role of neutral amino acids transporter (SLC7A10) and thiamine transporter (SLC19A3) in human primary adipocytes during thermogenic activation by cAMP is investigated more in details.

ASC-1 expression pattern in human neck derived adipocytes

Our RNA-Seq data showed that DN derived adipocytes expressed higher level of SLC7A10 as compared to SC derived adipocytes irrespective to differentiation protocols. The expression pattern of SLC7A10 was similar like UCP1 expression pattern and we could validate our RNA-Seq data by RT-qPCR using independent donors. We observed that the expression of ASC-1 was low in both SC and DN progenitors and was induced during differentiation in parallel with UCP1 expression levels. We did not notice any different level of ASC-1 expression between two anatomical origins at protein level but the expression of UCP1 was higher in DN as compared to SC adipocytes irrespective to differentiation protocols.

Facilitated serine, cysteine, and glycine uptake by DN adipocytes is hampered by ASC-1 inhibitor during thermogenic activation

To understand the functional importance of ASC-1 in human adipocytes, we measured the consumption of ASC-1 cargos including alanine, serine, cysteine, glycine, threonine, and glutamate by DN ADIPs and B-ADIPs upon thermogenic activation by cAMP for 10 h. We observed that serine, cysteine, glycine, and threonine were consumed at basal rate into unstimulated DN ADIPs and B-ADIPs. DN ADIPs consumed significantly higher amount of serine and cysteine as compared to B-ADIPs. Thermogenic activation by cAMP resulted in higher uptake of these amino acids in DN ADIPs and B-ADIPS and the uptake was hindered in the presence of specific noncompetitive ASC-1 inhibitor, BMS-466442. Intriguingly, alanine consumption into DN ADIPs was low and B-ADIPs even released alanine both in unstimulated and cAMP-stimulated conditions. Alanine release may indicate that B-ADIPs catabolize amino acids to provide sufficient fuels for TCA cycle and to convert nitrogen to pyruvate forming alanine as part of a possible glucose-alanine cycle in vivo [Felig, 1973]. ASC-1 preferentially facilitates the transport of neutral amino acids in an exchange mode, therefore, alanine release may allow the uptake of more cysteine, serine, and glycine. We also observed that DN ADIPs and B-ADIPs transported a significant amount of glutamate out and even higher amount of glutamate was released during thermogenic activation. These data suggested that active DN adipocytes consume higher amount of serine, cysteine, and glycine and ASC-1 mediates these amino acids uptake.

Inhibition of ASC-1 by its pharmacological inhibitor hampers UCP1-dependent and UCP1-independent oxygen consumption upon thermogenic activation

Next, we investigated the significance of ASC-1 in thermogenic activation of human adipocytes by measuring the OCR and observing the effect of ASC-1 inhibition by BMS-466442 during adrenergic stimulation. As expected, cAMP stimulation immediately elevated the OCR in ADIPs and B-ADIPS originated from both SC and DN (Figure 13a-b). The respiratory response of B-ADIPs was higher than ADIPs, both in SC and DN, and this observation was in line with higher expression of UCP1 and other thermogenic markers [Toth *et al*., 2020]. In addition, DN derived adipocytes showed higher OCR as compared to SC ones supporting the previous data describing

a higher thermogenic capacity of DN adipocytes. In unstimulated condition, the ASC-1 inhibitor significantly hampered cAMP-stimulated oxygen consumption of both SC and DN adipocytes. ASC-1 blocking did not affect the oxygen consumption rate of resting SC and DN ADIPs and B-ADIPs despite the reduced uptake of ASC-1 cargos in unstimulated adipocytes indicating that the ASC-1 contributes to cell respiration during thermogenic activation.

To investigate the effect of ASC-1 inhibition on UCP1-dependent portion of cellular respiration, oligomycin (ATP synthase blocker) was administered to the cells allowing us to estimate UCP1-dependent proton leak. We observed a decrease in UCP1-dependent proton leak of cAMP-stimulated SC and DN adipocytes indicating a significant influence of ASC-1 facilitated transport on mitochondrial proton gradient generation and its uncoupling. Contribution of a UCP1-independent respiration via creatine futile cycle to the cAMP-stimulated respiration was measured upon the injection of creatine analogue, β-GPA [Kazak *et al*., 2015]. B-GPA-inhibited portion of respiration was increased following cAMP stimulation and was abrogated by ASC-1 inhibitor in ADIPs and B-ADIPs irrespective to anatomical origins. Non-mitochondrial respiration, basal, and maximal ECAR were not affected by ASC-1 inhibitor. Our data pointed to the significance of ASC-1 in facilitating the uptake of amino acids needed for efficient thermogenic activation in human SC and DN derived adipocytes.

cAMP-stimulated increase of browning markers and mitochondrial complex subunits expression are hindered by ASC-1 inhibitor

Observing the effect of ASC-1 inhibition on the respiration of stimulated adipocytes, we presumed that cAMP-induced expression of thermogenic genes was also influenced. DN derived adipocytes had the tendency to have a higher expression of PGC1a, CKMT2, and UCP1 as compared to SC ones. As expected, cAMP increased the expression of those genes in ADIPs and B-ADIPs irrespective to anatomical origins (Figure 14). Inhibition of ASC-1 led to decrease cAMP-dependent upregulation of PGC1a, CKMT2, and UCP1 at both mRNA and protein levels.

We also investigated the effect of ASC-1 transporter inhibition on the mRNA expression of other brown/beige adipocyte markers as (Table 1) in SC and DN derived adipocytes. cAMP elevated the mRNA expression of CKMT1a/b, PM20D1, ELOVL3, DIO2, and CITED1 which was hindered by ASC-1 inhibition. The cAMP-induced elevation of TBX1 expression, which was halted by ASC-1 inhibitor, was observed only in DN ADIPs. Our data suggested that cAMP-induced upregulation of thermogenic genes in human neck derived adipocytes requires ASC-1 facilitated transport of serine, cysteine, and glycine.

In addition to thermogenic markers, we also investigated the influence of ASC-1 inhibitor on the protein expression of mitochondrial complex subunits. Thermogenic activation by cAMP elevated the expression of complex I and II subunits in DN ADIPs. We could not observe the cAMP-dependent upregulation in B-ADIPs since the basal expression of those complexes were already high. The cAMP-dependent upregulation of complex I, II, and IV was abrogated by ASC-1 inhibitor. The expression of complex III and V was not influenced either by cAMP or ASC-1

inhibitor (Figure 16d, f). Although it was not significant, we observed that SC ADIPs had lower expression of complex V.

UCP1 containing DN derived adipocytes expressed a high level of thiamine transporter 2

Among the 21 SLC transporters which were differentially expressed in SC and DN adipocytes, we found that the expression of ThTr2 (encoded by SLC19A3) was very low in preadipocytes and was robustly induced during differentiation. ThTr2 expression was influenced by anatomical origins (higher in DN as compared to SC derived adipocytes) and differentiation protocols (higher in B-ADIPs as compared to ADIPs) and RT-qPCR analysis using independent donors could validate the RNA-Seq data. Protein expression pattern of ThTr2 detected by immunoblotting were similar to the UCP1 indicating that ThTr2 was more expressed in high UCP1 containing DN derived adipocytes. ThTr1, encoded by SLC19A2, possessed lower expression and was not differently expressed between SC and DN derived adipocytes.

The expression of thiamine transporters was also checked in publicly accessible transcriptomic data set of adipocytes derived from human SC WAT (Min *et al*., 2019). ThTr2 expression was induced by differentiation and further elevated by adrenergic stimulation/forskolin while the expression of ThTr1 was not affected by either. In a thermogenic adipocytes cluster [Min *et al*., 2019], adrenergic stimulation increased the mRNA expression of both ThTrs. An RNA-Seq-based screening identified ThTr2 as adipose tissue-specific; its abundant expression in adipose tissue strongly correlated with the expression of mitochondrial genes suggesting a possible association between the expression of ThTr2 and brown/beige adipocytes (Pereira *et al*., 2021). Analysis of RNA-Seq data of Perdikari *et al*. (2018) reveals that ThTr2 expression is higher than ThTr1 expression (estimated by mRNA counts) in both human WAT and BAT obtained by needle biopsies from the neck area. The TPP transporter that facilitates the transport to mitochondria (TPC), encoded by SLC25A19, is expressed higher in BAT as compared to WAT. These data along with our observations (Fig.1) suggested that ThTr2 and thiamine might have a role in regulation of adipocyte thermogenesis.

Inhibition of ThTr2 hampered UCP1-dependent proton leak respiration

To investigate the importance of ThTr2 during thermogenic activation by cAMP in DN and SC derived adipocytes, we treated differentiated adipocytes with dibutyryl-cAMP (which mimics adrenergic stimulation in vivo) in the presence of a potent ThTr2 inhibitor, fedratinib [Zhang *et al*., 2014] and measured OCR in a regularly used culture medium which contains thiamine at 8.2 µM concentration. As anticipated, OCR was elevated immediately upon cAMP administration to DN derived ADIP while the response of SC derived ADIP was moderate. Fedratinib hindered the cAMP-stimulated elevation of OCR of ADIPs derived from both DN (Figure 19a, left and middle panels) and SC depots during the 10 hours monitoring. Proton leak respiration that mainly correlates with UCP1-dependent heat generation was calculated upon the injection of oligomycin (ATP synthase blocker). We found that cAMP-stimulated proton leak respiration was reduced by fedratinib in both DN and SC ADIPs.

Fedratinib can also inhibit tyrosine kinases including JAK2 [Zhang *et al*., 2014] and although tyrosine kinases are not known to be involved in adrenergic signaling of cultured adipocytes, we found it necessary to investigate the effect of other thiamine transporters inhibitor. We used thiamine analogue amprolium that inhibits the thiamine transport activity of both ThTr2 and ThTr1, with IC50 0.620±0.27 and 2.60±0.93 µM, respectively (Giacomini *et al*, 2017). Amprolium was also effective in significantly decreasing cAMP-stimulated maximal and proton leak respiration in both DN and SC derived ADIPs. cAMP-stimulated ECAR was not affected by the inhibition of ThTr2 by fedratinib in the adipocytes whereas amprolium treatment resulted in its decrease in DN ADIP.

This inhibitory effect of fedratinib was also observed in DN and SC adipocytes with more pronounced browning (B-ADIPs) resulted from their differentiation with constant PPARγ stimulation by rosiglitazone. cAMP elevated the OCR in both DN and SC B-ADIPs which was abrogated by fedratinib. Fedratinib did not influence the ECAR of either DN or SC B-ADIPs. The inhibitory effect of fedratinib and amprolium on OCR and proton leak respiration clearly show that continuous supply of thiamine through its transporters is needed for efficient thermogenic response of adipocytes.

Thiamine enhances thermogenic activation in DN and SC-derived adipocytes in a concentration-dependent manner

We went further to investigate the significance of thiamine availability during thermogenic activation of adipocytes using a thiamine free culture medium and co-injection of cAMP with gradually increasing concentrations of thiamine. In the absence of thiamine, as compared to 8.2 µM concentration present in regular culture medium, cAMP-stimulated OCR to maximal respiration rate was lower in both DN and SC ADIPs. cAMP-stimulated maximal respiration was increased already after addition of thiamine at 40 nM concentration (mild increase but significant) and elevated further at rising concentrations up to 25 µM. To elucidate the effect of increasing thiamine concentration on UCP1-dependent portion of cellular respiration, we calculated the OCR recorded following oligomycin administration and found that increasing thiamine concentration led to a higher proton leak respiration which could be observed already at the lowest used thiamine concentration, 40 nM. These results demonstrates that abundant thiamine availability have a critical importance during thermogenic activation of human adipocytes.

TPP enhances the activity of TPP-dependent PDH in permeabilized adipocytes

To further elucidate the mechanism of thermogenic action of thiamine, which is converted to the biochemically active compound TPP in cells, Seahorse-based respiration assay was optimized to monitor the activity of one of the TPP-dependent enzyme complexes, PDH, in cell membrane-permeabilized adipocytes which enabled us to study mitochondrial function without isolating mitochondria. Pyruvate and malate, which are substrates for PDH and intermediates in TCA cycle, were added to drive NADH generation. Upon the injection of membrane permeabilizing agent, OCR dropped in both DN and SC derived ADIPs. However, the difference in basal OCR between

DN and SC ADIPs as well as responsiveness to oligomycin were maintained under the permeabilized conditions. TPP addition significantly increased both maximal mitochondrial and proton leak respiration in DN derived ADIPs while a less pronounced effect was observed in SC ADIPs.

TPC was expressed higher in DN as compared to SC derived adipocytes and its expression was increased by cAMP indicating the elevated demand of TPP in the mitochondria during thermogenic activation. It is important to note that ThTr2 inhibition did not influence protein expression of TPC or PDHA1, and the latter was not affected by increasing thiamine availability either. The results obtained with the permeabilized adipocytes suggest that TPP-dependent PDH entities, which generate metabolic fuel for thermogenesis, are not fully saturated with bound TPP in differentiated adipocytes. This implies that excess thiamine converted to TPP in stimulated adipocytes can increase respiration and thermogenesis through elevating the level of TPP bound enzymes and thereby NADH production.

We also investigated the effect of inhibiting another component of the PDH complex, the E2 subunit, in intact adipocytes. We treated the cells with cAMP analogue in the presence of lipoic acid antagonist, devimistat [Zachar *et al*., 2011]. Devimistat inhibited cAMP-stimulated elevation of OCR in both DN and SC ADIPs at 1, 5, and 10 hours after injection (data are not shown). It increased ECAR in SC but did not affect that in DN ADIP (data are not shown). Importantly, proton leak respiration was decreased upon addition of devimistat (data are not shown) indicating that continuous availability of co-factors for steady-state activity of TPP dependent enzymes is critical for maintaining effective thermogenic stimulation.

Inhibition of thiamine transport led to a lower expression of thermogenic genes in SC and DN derived adipocytes

The hampered thermogenesis observed in the presence of ThTr inhibitors raised the possibility that limited thiamine availability could influence thermogenic gene expression. As described in the previous chapter, DN ADIPs expressed higher level of UCP1 and PGC1a as compared to SC adipocytes. cAMP elevated mRNA and protein expression of these thermogenic marker genes in both SC and DN ADIPs at thiamine concentration present in the regular culture medium. Inhibition of ThTrs by either fedratinib or amprolium resulted in attenuated cAMP-dependent upregulation of UCP1 and PGC1a. Furthermore, the high expression of both UCP1 and PGC1a in DN ADIP was reduced as a result of inhibitor treatments even in unstimulated condition suggesting that continuous supply of thiamine is required to maintain a high basal expression of UCP1 and PGC1a.

We also investigated the effect of fedratinib on other brown/beige markers. The cAMP-stimulated upregulation of thermogenic genes such as DIO2, CITED1, CIDEA, and TBX1 was abrogated in response to fedratinib during thermogenic activation in both SC and DN ADIPs.

We also investigated the effect of fedratinib on thermogenic gene expression in SC and DN B-ADIPs. We found that cAMP could not induce further the upregulation of UCP1 and PGC1a as their basal expression in B-ADIP was already high. However, we observed that ThTr2 inhibition resulted in the decreased expression of UCP1 and PGC1a. In addition, we found that the expression

of DIO2 and TBX1 was also hindered by fedratinib treatment demonstrating that thiamine transport is required for maintaining the high expression of thermogenic genes in thermogenically active B-ADIPs as well.
Next, we addressed the question whether inhibition of ThTr2 affects the protein expression of thermogenic markers and ThTrs in situ. Therefore, we dissected pairs of SC and DN biopsies into three pieces which were incubated in culture media with 8.2 µM thiamine. The first sample served as a control, the second was treated with cAMP analogue, and the third one was co-treated with the cAMP analogue and fedratinib or amprolium. In accordance with the literature and our observations ex vivo, UCP1 and PGC1a was expressed at a higher level in DN compared to SC biopsies. UCP1 and PGC1a was remarkably upregulated in response to cAMP-dependent activation, which effect was significantly inhibited by fedratinib and amprolium in both SC and DN adipose tissues.

Induction of thermogenic gene expression by thiamine in a concentration-dependent manner
As we observed that there was an increase of proton leak respiration following a gradually increasing concentration of thiamine, we hypothesized that thiamine availability also regulated the expression of thermogenic markers. Using thiamine free culture fluid, we could observe that cAMP-stimulated upregulation of UCP1 and PGC1a were potentiated by applying increasing concentrations of thiamine. Thiamine potentiated the expression of UCP1 at 40 nM and 1 µM in SC and DN ADIPs, respectively. The effect of thiamine on PGC1a expression was already observed at 40 nM in both SC and DN ADIPs. The influence of thiamine concentration on other thermogenic markers was also investigated. We found that thiamine potentiated cAMP-stimulated upregulation at its low concentration. These results indicated that thiamine could potentiate the cAMP-stimulated expression of thermogenic genes.

DISCUSSION

Human DN derived adipocytes possess an overlap molecular signature of classical brown and beige adipocyte markers

Hitherto, there is discrepancy regarding the molecular identity of human BAT, particularly BAT which is located in supraclavicular and cervical region. We addressed a question whether human adipocytes derived from DN possess classical brown adipocyte identity or it is more resemble to beige adipocyte. We characterized human neck derived adipocytes by performing global transcriptomic analysis and analyzed the expression pattern of established markers for classical brown and beige adipocytes. The differentiated adipocytes derived from two anatomical origins, SC and DN depots, showed a similar expression pattern of general adipocytes such as LEP, AIDPOQ, LEP, LEPR, and FABP4 indicating that the adipogenic program can be applied to hASCs derived from both anatomical origins and the differentiation rate of adipocytes originated from SC and DN are equal.

When we investigated the expression pattern of classical brown and beige adipocyte markers, we found that those markers were differently expressed between SC and DN. Similar to other studies in human supraclavicular and deep neck, we found that the expression of UCP1 was higher in DN than SC adipocytes. The expression of EBF2 which determine the brown adipocyte commitment was already high in DN preadipocyte and its high expression was maintained after differentiation. The expression of one of classical brown adipocyte marker, LHX8, and beige markers such as CIDEA, CITED1, PM20D1 KCNK3, and TBX1 was significantly higher in DN adipocytes. The expression of CIDEA, CITED1, and PM20D1 was also upregulated by rosiglitazone irrespective to anatomical origins. Intriguingly, well-known thermogenic markers such as PGC1a and ELOVL3 were not differently expressed between SC and derived adipocytes. Our results suggest that hASCs isolated from DN tissues consist of a mixture of progenitors which have potential to differentiate to classical brown or beige adipocytes.

In line with higher expression of thermogenic markers, DN derived adipocytes also possess higher brown adipocyte content and browning capacity score measured by BATLAS and ProFAT, respectively [Perdikari *et al*., 2018; Cheng *et al*., 2018]. Regardless the anatomical origins, rosiglitazone increased the BATLAS and ProFAT score. DN B-ADIPs had the highest BATLAS and ProFAT scores among all groups indicating that high thermogenic capacity in DN derived adipocytes can be induced further. The response of adipocytes derived from SC tissue, which is marked by higher expression of beige markers and score of BATLAS and ProFAT, indicate that masked beige adipocyte are present and can be induced by rosiglitazone or beta-adrenergic stimulation.

The expression of SLC transporters facilitating the uptake of cellular metabolic substrates is upregulated in thermogenic adipocytes

SLC transporters comprise a dynamic work for living cells by transporting essential nutrients including macronutrients, such as glucose, fatty acids, and amino acids, and micronutrients, such as vitamins and minerals [Zhang *et al*., 2019]. Active brown/beige adipocytes consume higher

amounts of nutrients to provide sufficient fuel for heat generation and SLC transporters play a crucial role in mediating the transport of those molecules. The uptake of glucose in adipocyte is primarily mediated by GLUT4 (encoded by SLC2A4) [El Bacha *et al*., 2010]. GLUT4 has been regarded as the pre-dominant insulin-dependent glucose transporter for decades until a study reported that there was another insulin-sensitive glucose transporter GLUT12, which is encoded by SLC2A12. Like GLUT4, GLUT12 translocate to cell membrane upon insulin stimulation. Our RNA-Seq analysis showed that the expression of GLUT12 was significantly higher in DN as compared to SC adipocytes. As we found that expression of GLUT4 is similar between SC and DN adipocytes, the upregulation of GLUT12 in DN adipocytes indicate that DN adipocytes require an additional facilitated-glucose transport to fulfill a higher energy demand. In the future, a functional study is needed to uncover the importance of GLUT12 during thermogenic activation in human adipocytes.

We found that one of fatty acid transporter family, FATP3 (encoded by SLC27A3), was expressed higher in DN than SC adipocytes and long-term rosiglitazone treatment increased its expression. The studies reported the function and cellular location of FATP3 are still limited. FATP3 plays a critical role in mediating fatty acids uptake in endothelial cells [Kazantzis and Stahl, 2012], however its function in other tissues or its role in pathological condition remain unclear. The expression of FATP3 was significantly reduced in human abdominal adipose tissue biopsies upon 5-week calorie restriction [Bouwman *et al*., 2014]. FATP3 can localize to mitochondria [Pei *et al*., 2004] and has acyl-CoA ligase activity for long-chain and very-long-chain fatty acids. The function of FATP3 in human adipocyte during thermogenic activation was still unknown. The upregulation of FATP3 in thermogenic adipocytes may be related to UCP1 activation by long-chain fatty acids [Fedorenko *et al*., 2012; Bertholet and Kirichok, 2017].

In addition to macronutrients transporters, SLC transporters that mediate the uptake of thiamine (described more detail in later chapter) and folic acid (encoded by SLC46A1) were also expressed higher in DN than SC adipocytes. Folic acid, in its active form tetrahydrofolate (THF) plays role in transporting single-carbon group which will be transferred to other molecules as part of biosynthesis process of biological molecules. The expression of Na/K/Ca exchanger NCKX3, encoded SLC24A3 respectively, were also higher in DN adipocytes as compared to SC adipocytes while NCKX2 encoded by SLC24A2 was expressed higher in B-ADIPs. These ion exchangers transport 1 Ca2+ and 1 K+ in exchange for 4 Na+. Calcium cycling that involves ryanodine receptor (RyR) and SERCA1 is one of the UCP1-independent thermogenesis mechanism [Ikeda *et al*., 2017]. Further investigation is needed to confirm the involvement of calcium transporter in UCP1-independent thermogenesis by calcium cycling.

The importance of ASC-1 facilitated amino acids uptake on thermogenic response of human neck adipocytes

Our data showed that ASC-1 was expressed higher in thermogenic adipocytes, similarly to UCP1. At the protein level, ASC-1 expression was moderately higher in ADIPs as compared to B-ADIPs irrespective to anatomical origins. Our finding was in line with other human study using tissue

samples obtained by need biopsies that found a slightly higher ASC-1 mRNA expressed in SC white tissue as compared to DN tissue [Perdikari *et al*., 2018]. Previous studies identified ASC-1 as cell surface markers of white adipocytes in human and mice [Ussar *et al*, 2014; Garcia *et al*, 2016]. Interestingly, ASC-1 mRNA is expressed at the same level in white and brown adipocytes isolated from SC and DN after immortalization sug-gesting that a loss of repression of ASC-1 occur in immortalized adipocytes [Ussar *et al*., 2014]. In mice, ASC-1 mRNA expression is less in beige adipocytes (differentiation under rosiglitazone) as compared to white adipocytes. The presence of ASC-1 in mice subpopulation of SC preadipo-cytes of adolescent adipose tissue and in differentiating preadipocyte cell line resulted in the in-hibition of beige differentiation program [Suwandhi *et al*., 2021].

We have investigated the role of ASC-1 in facilitating the uptake of serine, cysteine, and glycine in the metabolism and regulation of thermogenic adipocytes. Our metabolomics data showed that DN adipocytes consumed higher amount of serine, cysteine and glycine during thermogenic activation and ASC-1 played an important role in maintaining these amino acids in-flux. Inhibition of ASC-1 resulted in reduced uptake of these amino acids in basal and cAMP-stimulated condition in DN adipocytes. Serine is an important metabolic source to generate one-carbon unit in mammalian cells [de Koning *et al*., 2003]. One-carbon units are generated through break down of serine by both serine hydroxymethyltransferase (SHMT) enzymes, SHMT1 and SHMT2. One-carbon unit metabolism form a functional interaction with mitochondrial oxidative phosphorylation (OXPHOS) system that is crucial for ATP generation in mammalian cells. In our study, we have shown that mitochondrial OXPHOS system was affected by ASC-1 activity modulation as cAMP-induced elevation of mitochondrial complex I and II protein expression was alleviated by ASC-1 inhibitor. The disturbance of serine influx that leads to the reduction of one-carbon unit level may be one of major factor causing the decreasing of mitochondrial complex protein expression. One-carbon units are also required for shifting the methylation to trans-sulfuration pathway during glutathione (GSH) synthesis as a response to oxidative stress [McCarthy *et al*., 2014]. Serine treatment in mice diminishes oxidative stress by enhancing GSH synthesis and lowering hepatic reactive oxygen species (ROS) production [Zhou *et al*., 2017].

We investigated the significance of ASC-1 transporter in cAMP-stimulated OCR of human neck derived adipocytes in the presence of specific non-competitive ASC-1 inhibitor BMS-466442. In unstimulated condition, BMS-466442 did not affect the OCR of both SC and DN adipocytes. The hampering of ASC-1 activity led to the alleviation of cAMP-stimulated OCR in both SC and DN adipocytes. We observed that UCP1-dependent proton leak respiration, which was monitored upon oligomycin injection, was reduced during thermogenic activation in the presence of ASC-1 inhibitor. These data demonstrate the significance of ASC-1-mediated amino acid uptake for efficient thermogenic response of hu-man neck adipocytes. mRNA expression of ASC-1 is higher in abdominal SC tissue from lean as compared to obese individuals [Ussar *et al*., 2014; Jersin *et al*., 2021]. Serine catabolism also plays a crucial role in sustaining mitochondrial respiration [Lucas *et al*., 2018]. We also observed that the interruption of serine influx ablated the cAMP-

induced increasing of oxygen consumption at maximal respiration and UCP1-dependent respiration.

Higher expression of ASC-1 may give an advantageous effect for thermogenic activation of masked beige adipocytes residing in abdominal SC tissue of lean individuals. In line with OCR data, cAMP-stimulated mRNA and protein expression of thermogenic regulators such as UCP1 and PGC1a was also reduced by ASC-1 inhibitor in both human SC and DN adipocytes. Furthermore, we also found that the cAMP-stimulated mRNA expression of ELOVL3, CITED1 and DIO2 in human neck-derived adipocytes was reduced by the ASC-1 inhibitor (Fig-ure 15). This data once again invigorates the significance of ASC-1 on thermogenic activation in human neck-derived adipocytes.

The inhibition of ASC-1 also hampered the activation of creatine futile cycle, which is of the mechanism in the UCP1-independent thermogenesis. We observed a decrease in β-GPA-inhibited OCR and CKMT2 expression in both SC and DN adipocytes. In addition, cAMP-induced mRNA expression of CKMT1a/b was also reduced by the ASC-1 inhibitor. CKMT1a/b, the mitochondrial creatine kinase 1a/b, phosphorylates the creatine generating the phosphocreatine. CKMT1a/b plays an important essential role in UCP1-independent thermogenesis through the creatine futile cycle [Kazak *et al*., 2015]. Three amino acids including methionine, glycine and arginine are required to synthesize creatine [Da Silva *et al*., 2009]. The interruption of glycine influx by ASC-1 inhibitor may reduce the creatine synthesis leading to the decreasing of creatine futile cycle rate and creatine kinase expression. In addition to the creatine futile cycle, ASC-1 inhibition also reduced cAMP-stimulated mRNA expression of PM20D1 in human SC and DN adipocytes. PM20D1 was enriched in classical BAT as compared to epididymal WAT and PM20D1 administration increase the energy expenditure [Long *et al*., 2016]. PM20D1 regulates the synthesis of N-lipidated/N-fatty-acyl amino acids which function as endogenous uncouplers of mitochondrial respiration in a UCP1-independent manner.

ASC-1 has been recently identified as a novel regulator of energy metabolism reporting that ASC-1 overexpression reduces ROS formation, lipid accumulation, and insulin resistance and enhances mitochondrial respiration in human abdominal subcutaneous adipocytes [Jersin *et al*., 2021]. The important role of ASC-1 in controlling obesity has been also highlighted by the obser-vation in multiple cohorts that its expression in visceral adipose tissue is inversely correlated with insulin resistance and adiposity [Jersin *et al*., 2021]. The capacity to dissipate energy as heat is lower in obese individuals [Jung *et al*., 1979; Rosenbaum and Leibel, 2010]. Based on our pre-sented data, it can be presumed that decreased expression of ASC-1 in hypertrophic WAT of obese individuals may partially contribute to attenuated thermogenic response. This also might partially explain the fact that obese individuals possess high amounts of brownable but thermogenically inactive fat [Leitner *et al*., 2017]. Our findings suggested that stimulation of ASC-1 expression in adipose tissue of obese patients may have a beneficial effect on energy homeostasis and may be a promising therapeutic target for obesity and associated metabolic disturbance.

Abundant thiamine availability and its uptake by ThTrs is important for the effective thermogenic activation in human neck derived adipocytes

High energy demand in active brown/beige adipocytes might require the upregulation of SLC transporters to maintain an adequate influx of nutrients required for energy production. ThTr2 (encoded by SLC19A3) is one of the SLC transporters that were upregulated in DN adipocytes. ThTr2 expression was induced during the differentiation and was higher in DN as compared to SC adipocytes. Based on integrative analysis utilizing human transcriptomic and GWAS database, ThTr2 was identified as a novel human adipose-specific gene [Ahn *et al*., 2019]. A recent report demonstrated that SC adipose tissue isolated from individuals with obesity expressed lower ThTr2 as compared to lean individuals and the expression of ThTr2 positively correlated with weight loss [Pereira *et al*., 2021] suggesting its possible role in augmentation of energy metabolism. An in vivo study showed that ThTr2-deficient mice had decreased uptake of thiamine in intestine. However, ThTr1-deficient mice did not show a reduced thiamine uptake suggesting that ThTr2 can compensate for the loss of ThTr1. In addition, ThTr2 expression was upregulated in the intestine of ThTr1-deficient mice underlying the importance of the presence of ThTr2 in normal uptake of thiamine in mice [Reidling *et al*., 2010]. In humans, ThTr2 is postulated to play a major role in intestinal absorption because deficiency of ThTr1 does not alter plasma thiamine levels [Marce-Grau *et al*., 2019]. We observed that ThTr1 expression was in-duced during differentiation, however, we did not detect any different expression level between SC and DN adipocytes. The expression of both ThTrs was elevated by forskolin but this response can only be observed in thermogenic cluster adipocytes derived from abdominal SC (Figure 18c, d, [Min *et al*., 2019]). In human neck tissues obtained by needle biopsies, we observed that both ThTrs were expressed at the same level in WAT and BAT [Perdikari *et al*., 2018].

Thiamine, or vitamin B1, is transported into cells by ThTr and subsequently converted to the active form, TPP, by TPK. Several enzymes such as TK, a subunit of PDH, α-KGDH, and BCKDH complexes require TPP as their cofactors. As it was enriched in UCP1-containing thermogenic adipocytes, we intended to reveal the importance of ThTr2 during thermogenic activation in human neck derived adipocytes. Blocking of ThTr2 activity by its potent inhibitor, fedratinib, dampened the maximal and proton leak respiration of adipocytes stimulated for heat production. We could not exclude the fact that fedratinib is also a potent inhibitor of JAK2 and has been widely used in cancer therapy. This brings up the possibility that the observed effects of fedratinib on thermogenic activation might be resulted from the inhibition of an activated tyrosine kinase, especially in view that JAK2 KO mice were unable to upregulate BAT UCP1 following a HFD or after cold exposure [Shi *et al*., 2016]. However, involvement of JAK2 in BAT regulation could be demonstrated only in the presence of non-adipocyte tissue cells (which is not the case in our ex vivo differentiated adipocytes) and it was dispensable for induction of UCP1 thermogenesis in white adipose tissue through beige adipocytes which are presumed to be dominant among DN adipocytes. Nevertheless, we decided to perform all critical experiments using a competitive general thiamine transporter inhibitor, amprolium which also exerted comparable effect like fedratinib. To the best of our knowledge, we are the first group reporting the significance of ThTrs activity during thermogenic

activation in human adipocytes. The available thiamine in the culture media also affected maximal and proton leak respiration of human neck derived adipocytes in a concentration-dependent manner. The lack of thiamine halted the cAMP-stimulated elevation of maximal and proton leak respiration of the adipocytes. Our data suggest that thermogenically active brown/beige adipocytes require high amounts of thiamine taken up from the extracellular space majorly via ThTr2.

Cells with a greater energetic demand, such as active brown/beige adipocytes might need extra amount of thiamine to boost the activity of TPP-requiring enzymes that are responsible for energy metabolism. We elucidated the effect of direct stimulation by TPP supplementation on PDH activity in cell membrane-permeabilized adipocytes. TPP treatment increased the mitochondrial and proton leak respiration in the mitochondria within a special buffer that contained pyruvate and malate as substrates for energy production. We speculated that PDH, which is a key enzyme complex in catabolism, is initially not fully saturated by TPP in adipocytes that have not been activated for thermogenesis. The expression of mitochondrial TPP trans-porter/TPC was higher in thermogenic adipocytes and was further elevated by cAMP. Other study reported that high-dose thiamine therapy improved dyslipidemia in streptozotocin-induced diabetic rats by elevating the expression and saturation of TK [Babaei-Jadidi *et al*., 2004]. TPP treatment also elevated oxygen consumption rate and ATP turnover of mouse brain mitochondria upon cardiopulmonary resuscitation [Ikeda *et al*., 2016].

We were surprised when we found that thiamine availability could be connected to the regulation of thermogenic genes. Inhibition of thiamine transport into stimulated adipocytes led to decreased expression of UCP1 and other thermogenic regulators upon adrenergic stimulation. Addition of thiamine in thiamine free culture condition to the stimulated adipocytes could increase the expression of these genes in a concentration dependent manner. We can only speculate about the mechanism of how thiamine may contribute to induction of thermogenic genes during adrenergic stimulation of adipocytes. The 5´ non-coding region of the UCP1 gene contains regulatory elements that confer tissue specificity, differentiation dependence, and neuro-hormonal regulation to UCP1 gene transcription through interactions with a large number of transcription regulators including cAMP-responsive transcription factors as well as the PGC-1a co-regulator [Villaroya *et al*., 2017]. Transcriptional regulation of the UCP1 gene by cAMP-mediated signaling is provided by protein kinase A mediated rapid phosphorylation of the CREB transcription factor at the proximal UCP1 promoter region and p38 MAP kinase-mediated phosphorylation of ATF2 at the upstream enhancer region constituting a fast mechanism of regulation [Robidoux et a., 2005]. Thiamine or TPP may directly affect this orchestrated transcriptional regulation of UCP1 and other thermogenic regulators at these complex regulatory sites. Previous study reported that thiamine inhibits p53 DNA binding in living cells [McLure *et al*., 2004]. Phosphorylation mediated or TPP dependent metabolic changes occurring during thermogenesis may also generate so far not identified, indirect gene regulatory signals.

Clinical significance

Thiamine deficiency, which is mainly caused by inadequate nutrition intake and defects in thiamine transporters, is a major factor of several diseases such as beriberi or Wernicke's encephalopathy and Korsakoff psychosis referred to as Wernicke-Korsakoff syndrome (WKS) [Cook *et al*., 1998]. The main symptoms of WKS are memory impairment, ataxia, confabulation, and hypothermia which is frequently reported as secondary symptom. Under normal physiological and nutritional conditions, a healthy human adult has approximately a 3-weeks reserve of thiamine in the liver [Maguire *et al*., 2018]. These reserves are rapidly depleted in chronic alcohol consumption that leads to disturbance of thiamine absorption [Cook *et al*., 1998; Cruickshank *et al*., 1988; Donnino *et al*., 2010]. It has been reported that thiamine deficiency leads to the lesion of the hypothalamus, which is the main regulator of body temperature and appetite [Tanev *et al*., 2008]. Human case studies have reported that parenteral administration of thiamine improved the hypothermic condition after 2 days of treatment [Hansen *et al*., 1984]. Our presented data suggest that in addition to causing disturbance in hypothalamic thermoregulation, thiamine deficiency may also compromise peripheral thermogenesis in brown/beige adipocytes contributing to hypothermia in WKS patients.

Alcohol consumption positively correlates with visceral fat accumulation in healthy individuals [Kim *et al*., 2012; Dorn *et al*., 2003], which may be partially caused by the perturbance of thiamine absorption and metabolism. Thiamine deficiency has also been reported in type 1 and 2 diabetes patients [Thornalley *et al*., 2007]. Thiamine supplementation (100 mg, 3x100 mg daily which is about 100x higher than recommended daily allowance) for 6 weeks improves glucose tolerance in hyperglycemic individuals [Alaei *et al*., 2013]. It has also been reported that thiamine supplementation may provide beneficial effects in type 2 diabetes patients by improving lipid and creatinine profiles [Al-Attas *et al*., 2014]. Obese individuals exerted significant thiamine deficiency before undergoing bariatric surgery [Carrodeguas *et al*., 2005; Flanchbaum *et al*., 2006; Nath *et al*., 2017; Peterson *et al*., 2016; Aron-Wisnewsky *et al*., 2016]. A recent study reported that subcutaneous adipose tissue isolated from obese individuals expressed lower ThTr2 as compared to lean individuals. The expression of ThTr2 in subcutaneous adipose tissue was positively correlated with weight loss [Pereira *et al*., 2021] suggesting its possible role in augmentation of energy metabolism. A recent report demonstrated that SC adipose tissue isolated from individuals with obesity expressed lower ThTr2 as compared to lean individuals and the expression of ThTr2 positively correlated with weight loss [Pereira *et al*., 2021] suggesting its possible role in augmentation of energy metabolism. Thiamine supplementation prevented obesity and obesity-associated metabolic disorders in OLETF rats [Tanaka *et al*., 2010]. We have presented a functional ex vivo study revealing the importance of ThTr2, also ThTr1, during thermogenic activation and proposed the impact of thiamine availability in the regulation of thermogenic gene transcription. Our results raise the possibility to target molecular elements of the regulation of thiamine metabolism to augment heat generation by thermogenic adipocytes in obese individuals.

SUMMARY

Human adipose tissue stem cells (hASCs) were isolated from SC and DN tissues during thyroid surgery and differentiated the hASC using adipogenic differentiation protocols. RNA-Sequencing analysis was performed to compare the gene expression between SC and DN derived adipocytes. We found that DN derived adipocytes had higher expression of thermogenic genes such as UCP1, TBX1, CIDEA, CKMT1a/b, and PM20D1. They also possessed higher brown adipocyte content and browning degree quantified by BATLAS and ProFAT respectively. Among 1049 differentially expressed genes, we found 21 SLC transporters were differently regulated (15 of them were more expressed in DN adipocytes). Alanine-serine-cysteine transporter-1 (ASC-1) and thiamine transporter 2 (ThTr2) for further investigation.

ASC-1 mediates the transport of alanine, serine, cysteine, and glycine in a sodium-independent manner. In the presence of ASC-1 selective inhibitor, the uptake of these amino acids during thermogenic stimulation was dampened in both types of adipocytes. cAMP-stimulated oxygen consumption and proton leak respiration of subcutaneous and deep neck adipocytes was also reduced by ASC-1 inhibitor. Creatine futile cycle that drives UCP1-independent heat generation was also reduced during thermogenic activation in the presence of ASC-1 inhibitor. In accordance with oxygen consumption data, the expression of thermogenic markers (UCP1, CKMT1/2, PM20D1, ELOVL3, DIO2, and CITED1), mitochondrial biogenesis regulator (PGC1a), and mitochondrial complex subunits was hampered by ASC-1 inhibitor during thermogenic stimulation. Our data suggested that ASC-1 mediated serine, cysteine, and glycine uptake is required for the efficient thermogenic response upon cAMP stimulation in human neck derived adipocytes.

Thiamine is transported into the cells by ThTr1 (SLC19A2) and ThTr2 (SLC19A3). The inhibition of thiamine transport into the cells by potent inhibitors of ThTrs led to the reduced oxygen consumption and proton leak respiration during adrenergic stimulation. The importance of thiamine availability during thermogenic activation was also proven when we applied thiamine free culture fluid and gradually increased the thiamine concentration. We found that thiamine enhanced the thermogenic activation of human neck adipocytes in a concentration-dependent manner. The activity of pyruvate dehydrogenase, which is thiamine pyrophosphate-dependent enzyme, can be enhanced by directly stimulating the cell membrane-permeabilized adipocytes with thiamine pyrophosphate. The UCP1-dependent proton leak respiration of permeabilized adipocytes was elevated by thiamine pyrophosphate. This indicates that excess thiamine converted to thiamine pyrophosphate during thermogenic activation can increase mitochondrial respiration and thermogenesis by elevating the level of thiamine pyrophosphate bound enzymes and thereby NADH production. The expression of thermogenic markers during cAMP stimulation was also reduced when thiamine transport was inhibited by ThTrs potent inhibitors. In addition, we also found that thiamine potentiated the cAMP-stimulated thermogenic gene upregulation in a concentration-dependent manner. Thiamine deficiency, which is mainly caused by chronic alcohol consumption, leads to Wernicke-Korsakoff syndrome with hypothermia as a secondary symptom. Thiamine administration ameliorated the hypothermic condition suggesting the beneficial effect of thiamine in improving hypothalamic thermoregulation. Our presented data suggested that thiamine deficiency may also perturb peripheral thermogenesis in thermogenic adipocytes contributing to hypothermia of Wernicke-Korsakoff syndrome patients.

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Subject: PhD Publication List

Candidate: Rini Arianti
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List of publications related to the dissertation

1. **Arianti, R.**, Vinnai, B. Á., Bartáné Tóth, B., Shaw, A., Csősz, É., Vámos, A., Győry, F., Fischer-Posovszky, P., Wabitsch, M., Kristóf, E., Fésüs, L.: ASC-1 transporter-dependent amino acid uptake is required for the efficient thermogenic response of human adipocytes to adrenergic stimulation.
   FEBS Lett. 595 (16), 2085-2098, 2021.
   DOI: http://dx.doi.org/10.1002/1873-3468.14155
   IF: 4.124 (2020)

2. Bartáné Tóth, B., **Arianti, R.**, Shaw, A., Vámos, A., Veréb, Z., Póliska, S., Győry, F., Bacsó, Z., Fésüs, L., Kristóf, E.: FTO intronic SNP strongly influences human neck adipocyte browning determined by tissue and PPARγ specific regulation: a transcriptome analysis.
   Cells. 9 (4), E987, 2020.
   DOI: https://doi.org/10.1101/2020.02.21.959593
   IF: 6.6

List of other publications

3. Shaw, A., Bartáné Tóth, B., **Arianti, R.**, Csomós, I., Póliska, S., Vámos, A., Bacsó, Z., Győry, F., Fésüs, L., Kristóf, E.: BMP7 increases UCP1-dependent and independent thermogenesis with a unique gene expression program in human neck area derived adipocytes.
   Pharmaceuticals (Basel). 14 (11), 1-21, 2021.
   DOI: http://dx.doi.org/10.3390/ph14111078
   IF: 5.863 (2020)

4. **Arianti, R.**, Ariani, N. L., Muhammad, A. A., Sadewa, A. H., Farmawati, A., Sunarti, Hastuti, P., Kristóf, E.: Influence of Single Nucleotide Polymorphism of ENPP1 and ADIPOQ on Insulin Resistance and Obesity: a Case-Control Study in a Javanese Population.
   Life (Basel). 11 (6), 552-, 2021.
   DOI: http://dx.doi.org/10.3390/life11060552
   IF: 3.817 (2020)

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5. Shaw, A., Bartáné Tóth, B., Király, R., **Arianti, R.**, Csomós, I., Póliska, S., Vámos, A., Korponay-Szabó, I., Bacsó, Z., Győry, F., Fésüs, L., Kristóf, E.: Irisin stimulates the release of CXCL1 from differentiating human subcutaneous and deep-neck derived adipocytes via upregulation of NF[kappa]B pathway.
Front. Cell. Dev. Biol. 9, 1-19, 2021.
DOI: http://dx.doi.org/10.3389/fcell.2021.737872
IF: 6.684 (2020)

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