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Amino acids contribute to adaptive thermogenesis. New insights into the mechanisms of action of recent drugs for metabolic disorders are emerging

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ABSTRACT

Adaptive thermogenesis is the heat production by muscle contractions (shivering thermogenesis) or brown adipose tissue (BAT) and beige fat (non-shivering thermogenesis) in response to external stimuli, including cold exposure. BAT and beige fat communicate with peripheral organs and the brain through a variegate secretory and absorption processes – controlling adipokines, microRNAs, extracellular vesicles, and metabolites – and have received much attention as potential therapeutic targets for managing obesity-related disorders. The sympathetic nervous system and norepinephrine-releasing adipose tissue macrophages (ATM) activate uncoupling protein 1 (UCP1), expressed explicitly in brown and beige adipocytes, dissolving the electrochemical gradient and uncoupling tricarboxylic acid cycle and the electron transport chain from ATP production. Mounting evidence has attracted attention to the multiple effects of dietary and endogenously synthesised amino acids in BAT thermogenesis and metabolic phenotype in animals and humans. However, the mechanisms implicated in these processes have yet to be conclusively characterized. In the present review article, we aim to define the principal investigation areas in this context, including intestinal microbiota constitution, adipose autophagy modulation, and secretome and metabolic fluxes control, which lead to increased brown/beige thermogenesis. Finally, also based on our recent epicardial adipose tissue results, we summarise the evidence supporting the notion that the new dual and triple agonists of glucagon-like peptide-1 (GLP-1), glucose-

Abbreviations: Adipoq, adiponectin; AIF-1, allograft inflammatory factor 1; AMPK, AMP-dependent kinase; ANT, adenine nucleotide translocator; ARG1, arginase; Asn, asparagine; ATF4, activating transcription factor 4; ATG13, autophagy-related 13: ATGL, adipose triglyceride lipase; ATM, adipose tissue macrophages; β_3-AR, β_3-adrenergic receptor; BAT, brown adipose tissue; BCAA, branched-chain amino acids; BCAT2, branched-chain amino acid aminotransferase 2; BCKA, branched chain α-keto acids; BCKDH, branched-chain α-keto acid dehydrogenase; CAD, coronary artery disease; CABG, coronary artery bypass graft; CASTOR1, cytosolic arginine sensor for mTORC1 subunit 1; CB1, cannabinoid type 1 receptor; cAMP, 3',5'-cyclic AMP; CLS, crown-like structures; CoQ, coenzyme Q; CPT, carnitine palmitoyl-transferase; CKB, creatine kinase B; Cyt C, cytochrome C; Δμ, proton-based (H^+) electrochemical gradient; EAA, essential amino acids; EAT, epicardial adipose tissue; EE, energy expenditure; ETC, electron transport chain; FA, fatty acids; ^{18}F-FDG, ^{18}F-fluorodeoxyglucose; FGF21, fibroblast growth factor 21; FOXO3, forkhead box O3; ^{18}F-THA, ^{18}F-fluoro-thiaheptadecanoic acid; GATOR1, GAP activity towards the Rags 1; GCG, glucagon; GCN2, general control non-derepressable 2 kinase; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GCGR, glucagon receptor; GIPR, GIP receptor; GLP1R, GLP-1 receptor; HFD, high fat diet; HFD-EAA, amino acid substituted HFD; HSL, hormone-sensitive lipase; IFN-γ, interferon-γ; iNOS NOS2, inducible nitric oxide synthase; KISTOR, LPS, lipopolysaccharide; MAOA, monoamine oxidase; MITF, melanocyte-inducing transcription factor; NEAA, non-essential amino acids; mTORC1, mechanistic target of rapamycin complex 1; NST, non-shivering thermogenesis; NE, norepinephrine; NO, nitric oxide; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; PF, pair-feeding; PKA, protein kinase A; PKM2, pyruvate kinase M2; Pm20d1, peptidase M20 domain containing 1; PPARα, peroxisome proliferator-activated receptor α; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; Robo1, Roundabout receptor 1; SAM, S-adenosylmethionine; SAMs, sympathetic neuron-associated macrophages; SAMTOR, S-adenosylmethionine sensor; SDH, succinate dehydrogenase; Sesn2, Sestrin 2; SFA, saturated fatty acid diet; SFA-EAA, amino acid substituted SFA diet; SLC25, solute carrier family 25; Slc6a2, solute carrier family 6 member 2; Slit3, slit guidance ligand 3; SNS, sympathetic nervous system; TCA, tricarboxylic acid cycle; TFEB, transcription factor EB; TNAP, tissue-nonspecific alkaline phosphatase; TNF-α, tumor necrosis factor-α; T2D, type 2 diabetes; UCP1, uncoupling protein; ULK1, unc-51-like autophagy-activating kinase 1; VR, valve replacement; WAT, white adipose tissue.

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dependent insulinotropic polypeptide (GIP), and glucagon (GCG) receptor – with never before seen weight loss and insulin-sensitizing efficacy – promote thermogenic-like amino acid profiles in BAT with robust heat production and likely trigger sympathetic activation and adaptive thermogenesis by controlling amino acid metabolism and ATM expansion in BAT and beige fat.

1. Introduction

Adaptive thermogenesis indicates the heat production by the body in reaction to external stimuli, including low room temperature. It is considered crucial to the evolutionary success of mammals [1]. Generally, adaptive thermogenesis is classified into shivering (i.e., involuntary muscle contractions) and non-shivering thermogenesis (NST). Brown adipose tissue (BAT) has been identified as an important site of NST for decades and is critical for maintaining body temperature [2,3]. In rodents and human infants, this developmentally formed depot is abundant in the interscapular and perirenal regions. Additionally, distinct beige adipocytes appear in white fat depots also having a substantial ability to induce thermogenesis [4]. In the last 15 years, numerous research studies have provided compelling evidence regarding the presence of thermogenic adipose tissue in adult humans. This discovery has spurred further investigation into the mechanisms underlying heat production regulation. Two potential avenues for achieving this are: 1) enhancing the activity of existing brown adipose tissue (BAT) and 2) recruiting multiple thermogenic adipose populations that function effectively. These approaches encompass stimulating the differentiation of progenitor cells to generate new thermogenic adipocytes or reactivating dormant cells to reinstate the thermogenic phenotype. This suggests the existence of diverse progenitors and adipocyte populations that go beyond the conventional classification of brown and beige adipocytes [5]. A recent example of adipocyte heterogeneity involves the discovery of a unique subtype of beige adipocytes originating from myogenic PDGFRα^{+} progenitors near the lymph nodes of inguinal WAT depots. This specific subgroup of beige adipocytes, called g-beige fat, exhibits a distinct metabolic profile with a preference for glucose catabolism over fatty acids [6]. Interestingly, the induction of g-beige fat is mediated by non-adrenergic signaling pathways triggered by the nicotinic acetylcholine receptor subunit CHRNA2 downstream of acetylcholine produced by multiple immune cells within the inguinal WAT [7].

Another instance of thermogenic cell heterogeneity is observed in the interscapular BAT depots of mice. These depots contain two distinct subtypes of brown adipocytes considering adiponectin expression: Adipoq^{high} and Adipoq^{low}. The Adipoq^{low} brown adipocytes exhibit lower expression levels of thermogenic genes, larger lipid droplets, and reduced mitochondrial content compared to Adipoq^{high} cells [8]. Adipoq^{high} brown adipocytes increase in response to cold exposure but gradually decline in aged mice.

Furthermore, using single-nucleus RNA sequencing on BAT in mice and thermogenic fat depots in humans, a low-abundance population of adipocytes has been identified. These adipocytes negatively impact the thermogenic activity of neighboring brown adipocytes by increasing the local concentration of the short-chain fatty acid acetate [9].

The thermogenic fat depots drain directly into the systemic circulation, which may facilitate a more rapid flow of warmed blood throughout the body. Adult mice, a standard model for studies of vertebrate physiology, weigh 20–50 g, and BAT accounts for 2–5% of their body mass [10]. The entire WAT depot in lean adults ranges from 20 to 30 kg in women (30–40% of total body mass) and 10–20 kg in men (15–25% of total body mass). Because humans are three orders of magnitude larger than rodents, they require less BAT thermogenesis due to higher insulation from muscle and WAT and a lower surface area to volume ratio.

The distribution of energy is greatly influenced by fat tissue. Three macronutrients comprise all the food mammals consume: fats, proteins, and carbohydrates. The human body is capable of metabolic flexibility, which is the ability of mitochondria to alter their substrate preference for fat instead of carbohydrate oxidation [11]. Recent research has shown that dysfunctional mitochondria lead to insulin resistance in skeletal muscle, not because of impaired flexibility in response to substrate availability but because of an overall decrease in substrate oxidation [11]. Therefore, overfeeding with any of the macronutrients ultimately leads to a positive energy balance and weight gain [12]. As a result, the location of excess triglyceride storage is at least as important to metabolic health risks as the body's total fat mass [13]. WAT is typically divided into visceral and subcutaneous fat, which have different effects on metabolism – negative, neutral, or both [14]. Visceral fat can be divided into different parts, each with its metabolic risks.

Based on microscopic anatomy and cell physiology, it is more fitting to allude to fat as adipose tissue since it comprises numerous distinct cell types. The stromal vascular fraction, which includes fibroblasts, blood, and blood vessels, macrophages and other immune cells, nerve tissue, is also present, in addition to adipocytes. Numerous cell subtypes that play crucial roles in regulating adipogenesis, thermogenesis, and interorgan communication have been identified by relatively new technologies that involve single-cell and single-nucleus RNA sequencing [15,16]. Additionally, the various proteins and proteoglycans in the extracellular matrix play an active role in the function of both WAT and BAT [13,16].

In animal models of obesity and type 2 diabetes (T2D), the activation of a thermogenic program has been associated with an effective improvement in metabolic health, including weight loss, improved glucose homeostasis, and improved insulin sensitivity [17,18]. BAT activation prevents and treats insulin resistance in humans without affecting weight [19]. Brown fat's thermogenesis-independent mechanisms may also have to be considered [20]. As a result, BAT and beige adipose tissue have received much attention as potential therapeutic targets for managing obesity-related metabolic syndrome [21,22].

Studies using positron emission tomography-computed tomography (PET–CT) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG), a glucose tracer, ^{18}F-fluoro-thiaheptadecanoic acid (^{18}F-THA), a fatty acid tracer, and ^{18}F-fluciclovine, a leucine-analog tracer, have demonstrated that BAT also serves as a metabolic sink for glucose, fatty acids, and branched-chain amino acids (BCAA) [23–26]. The capacity to improve metabolic health is tightly linked to this function; by contributing to improved systemic lipid metabolism and systemic BCAA clearance, cold acclimatization stimulates BAT's uptake of glucose, triglyceride-rich lipoproteins, and fatty acids, in addition to BCAA [26–28]. A consistent body of literature sustains the potential therapeutic efficacy of several pharmacological or nutritional strategies to promote BAT thermogenesis and beige fat development. Amino acids' possible therapeutic effects on metabolic health and their role in promoting BAT adaptive thermogenesis promoted by novel anti-diabetic and anti-obesity drugs are discussed in depth in this review.

2. The role of amino acids in adaptive thermogenesis and its molecular mechanisms

2.1. NST and uncoupled respiration

To understand how amino acid combinations play a role in adaptive thermogenesis, one must consider thermodynamic principles and recent findings that identify adipose tissue heat producers. Traditional observations of mitochondrial uncoupling between respiration and ATP

synthesis in brown and beige adipose tissues have long intrigued researchers [29]. Understanding this phenomenon has been dramatically improved by identifying uncoupling protein 1 (UCP1) causes it [30]. Classically, oxidative metabolism is regulated by ATP requirement, and oxidative phosphorylation can relieve this respiratory constraint, as demonstrated with mitochondria isolated from multiple tissues. Despite the attempts to study respiratory control with isolated mitochondria of BAT, researchers were unsuccessful because the organelles were found "uncoupled" from oxidative phosphorylation. This result might explain the mitochondria's ability to oxidize substrates without restrictions, but it could also result in challenges in ATP supply during non-thermogenic conditions. According to the first experiments, mitochondria from brown fat demonstrated unusual permeability to chloride [31], suggesting the possibility of ion transport. Proton permeability also appeared to play a crucial role, suggesting that mitochondrial respiration in BAT could be explored in the context of Peter Mitchell's chemiosmotic hypothesis [32]. As Mitchell proposed to link the electron transport chain to the ATP synthase, the proton circuit could be quantified by determining its voltage and current (proton flux) and by applying Ohm's law [33] to calculate the conductance of the inner membrane to protons. A straightforward mechanism for controlling proton conductance appeared from those findings [34]. Indeed, fatty acids were found remarkably sensitive modulators of proton conductance – in the presence of purine nucleotides in cytosol – working at 30-fold lower concentrations than necessary for non-specific uncoupling [35]. A specific binding site for nucleotide was detected on the external surface of the inner membrane [36], and photo-affinity labeling identified this site as UCP1, a 32-kDa protein [37]. Further investigation has established that UCP1 is a member of the six transmembrane transporter family SLC25 on mitochondria [38].

2.2. Energy substrates and mitochondrial function

The tricarboxylic acid (TCA) cycle and its interaction with the electron transport chain (ETC), which consists of coenzyme Q (CoQ), cytochrome C (Cyt C), and complexes I through IV, are two systems that generate a proton-based (H^+) electrochemical gradient ($\Delta\mu$) that enables the cells to generate ATP through F_1F_0-ATPase in all mitochondria-containing cells. The sympathetic nervous system (SNS) activates the tissue-specific uncoupling protein 1 (UCP1) in brown and beige/brite adipocytes, dissolving the electrochemical gradient and uncoupling TCA and the ETC from ATP production. Instead, there is an increase in the rates of the associated exergonic reactions of TCA and the ETC, which cause heat to be produced [39]. This process is triggered by various stimuli, including cold exposure, diet consumption, thyroid hormones, and pharmacological treatment (*e.g.*, the β_3-adrenergic receptor agonists). In addition, Razzoli et al. proposed a new model of stress-induced sympathetic purinergic modulation of the BAT thermogenic program [40].

The sympathetic neurons release norepinephrine (NE) to activate adenylyl cyclase, with the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic AMP (cAMP), and activation of protein kinase A (PKA). This signal initiates a molecular cascade that triggers lipolysis via hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), including the mechanistic target of rapamycin (mTOR) signaling pathway, which is required for adipose browning by NE [41,42] (Fig. 1). The free fatty acids (FA) are transported into the mitochondrion via the carnitine palmitoyl-transferase (CPT) system and are oxidized via β-oxidation or directly activate UCP1 (Fig. 1).

The hydrolysis of ETC-produced ATP within the mitochondrial intermembrane space, commonly directing heat production, is one of the other thermogenic processes. This process is facilitated by the mitochondrial adenine nucleotide translocator (ANT), which transfers ATP from the mitochondrial matrix to the intermembrane space. At this site, creatine is transformed to phosphocreatine through mitochondrial creatine kinase B (CKB) and finally hydrolyzed by tissue-nonspecific

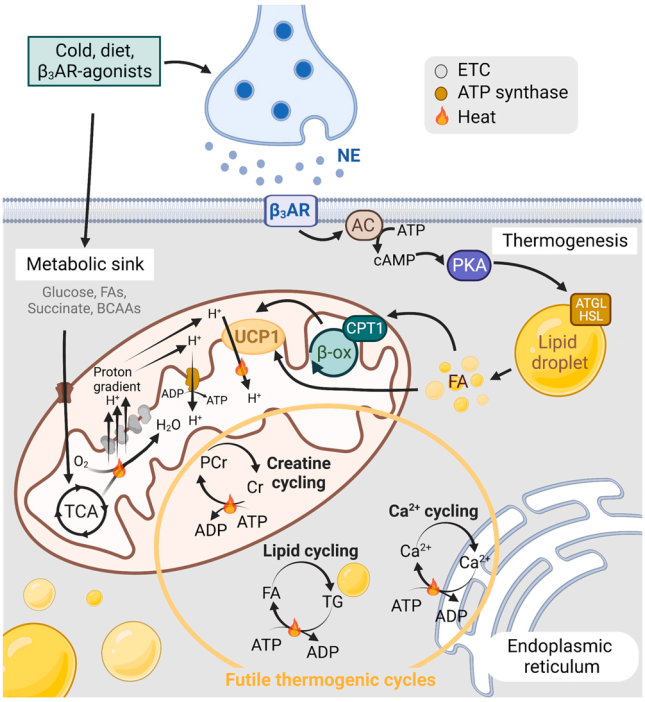

Fig. 1. Metabolic control of adipose thermogenesis. Adipose thermogenesis (e.g., the energy lost as heat through the metabolism of macronutrients in specialized adipocytes) is activated by neuronal and metabolic stimuli in response to cold exposure, diet consumption, or pharmacological treatment (*e.g.*, β_3-AR agonist). The sympathetic neurons (SNS) release norepinephrine (NE) to activate adenylyl cyclase (AC), with the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic AMP (cAMP) and activation of protein kinase A (PKA). This initiates a signalling cascade that triggers lipolysis via hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). The free fatty acids (FAs) are transported into the mitochondrion via the carnitine palmitoyltransferase (CPT) system, and they are oxidized to generate a proton gradient via β-oxidation or directly activate uncoupling protein 1 (UCP1). Mitochondrial protein UCP1 can short-circuit this proton gradient, providing reducing equivalents for the electron transport chain (ETC) and dissipating energy as heat. The high catabolic metabolism, through UCP1 activation and futile cycling (i.e., creatine, lipid, and calcium cycling), drives demand for reduced ETC substrates from intermediates of the tricarboxylic acid (TCA) cycle. Once activated, thermogenic adipocytes are metabolic sinks that consume macronutrients, such as glucose, FAs, succinate, and branched-chain amino acids (BCAAs), from the blood. Beyond activating UCP1, the metabolites and FAs fuel the ETC and TCA cycle to replenish the proton gradient driving ATP synthesis. Increasing flow through the TCA cycle and ETC is the basis of thermogenesis. Created using BioRender.com.

alkaline phosphatase (TNAP), leading to the generation of heat through the superstoichiometric futile cycling (Fig. 1; additional futile cycles are reported in the legend). This UCP1-independent thermogenetic process has been recently put into discussion [43]. Conceptual inconsistency, lack of conclusively supportive experiments of BAT mitochondrial respiration, and the constraints of the low ATP synthase activity and thermodynamic characteristics of brown adipocytes are some of the critical issues against a futile creatine cycle supporting NST.

An alternative thermogenic process has been proposed in brown fat. Succinate dehydrogenase (SDH), a component of ETC complex II and one of the enzymes of the TCA cycle, oxidizes succinate and produces reactive oxygen species that drive thermogenic respiration [44]. Additionally, N-acyl amino acids – derived from the condensation of FFAs and amino acids by the cold-inducible secreted enzyme peptidase M20 domain containing 1 (Pm20d1) – have been found recently to activate mitochondrial uncoupling respiration in a UCP1-independent manner

and increase energy expenditure (EE) in vivo [45]. In contrast, some results sustain that it is improbable that the N-acyl amino acids are of specific physiological relevance as UCP1-independent thermogenic compounds [46]. Thus, these new UCP1-independent mechanisms, proposed to play a major role in brown and beige adipose tissue NST, must be subjected to detailed scrutiny.

Multiple pieces of evidence support essential amino acids (EAA) and non-EAA controlling BAT thermogenesis. According to Yoneshiro et al. [26], systemic glucose intolerance and decreased thermogenesis result from a BAT-specific ablation of the BCAAs transporter into mitochondria. To prevent hypothermia caused by anesthesia, an amino acid mixture is administered intravenously [47]. According to Yamaoka [48], other amino acids may also play a role in attenuating hypothermia, even though BCAAs are necessary but insufficient for this purpose. Xu et al. demonstrated that asparagine (Asn), a non-EAA that becomes essential for tumor cells when glutamine is depleted [49,50], reinforces mTORC1 signaling to boost thermogenesis in adipose tissues [51]. Circulating Asn levels are inversely associated with metabolic syndrome [52] and obesity in both adults [53] and children [54]; additionally, acute cold exposure and cold acclimation significantly increased BAT Asn and other amino acid levels in mice [55], as well Asn feeding potentiates sensitivity to β_3-adrenergic receptor agonist CL316,243 on body weight and more significantly systemic insulin sensitivity [51]. It is common knowledge that distinct nutrient-related responses may result from varying amounts of amino acids in the diet. According to multiple reports, glutamine replenishes α-ketoglutarate for the TCA cycle or maintains fatty acid synthesis by reductive carboxylation to form citrate, particularly in low-oxygen conditions [56–58]. O-linked N-acetylglucosamine modification was found to be associated with an activated pro-inflammatory pathway and lower glutamine levels in WAT from obese individuals [59]. Alanine gavage to lean or diet-induced obese mice improved glucose tolerance by robustly boosting the AMP-dependent kinase (AMPK) signaling cascade in skeletal muscles [60]. Serine binds and activates pyruvate kinase M2 (PKM2) to control glycolytic flux in cancer cells [61]; it also supports mitochondrial dynamics, respiration, and translation by the generation of ceramide or N-formylmethionine-tRNA [62–64].

2.3. Signaling properties of amino acids in thermogenic fat

Traditional views of amino acids as simple backbones for protein synthesis were outdated and replaced by an integrated view supporting amino acids and their metabolites/intermediates as signaling molecules, either through effects on metabolic pathways or via modulation of other regulatory proteins. Recent investigation has also uncovered novel roles for several amino acids and metabolites that expand their signaling influence to processes outside metabolism, including nutrient sensing and storage, embryonic development, cell survival and differentiation, and immune activation and cytokine secretion. As very opportunely stated by Baker and Rutter in a recent review to which we refer the interested readers, “these studies suggest that, in contrast to the prevailing notion, the biochemistry of a cell is frequently governed by its underlying metabolism rather than vice versa.” [65].

As we will discuss in the paragraph dedicated to the role of autophagy in BAT and beige fat thermogenesis, amino acids are critical signals to regulate mTORC1 activity and energy homeostasis. A major metabolic inhibitor of mTORC1 is AMPK, which is potently activated by AMP [66], providing a mechanism to inhibit mTORC1 activity in the setting of low energy. While mTORC1 responses are optimised to sense amino acid sufficiency, the GCN2 (general control non-derepressable 2)/ATF4 system in mammalian cells has evolved to sense an amino acid restriction or, more precisely, amino acid imbalance [67–69]. Its main consequences are to reduce global translation and, at the same time to increase the cellular amino acid pool through increasing biosynthesis and amino acid transport. Any depletion of a particular amino acid will eventually result in unloaded tRNAs. Uncharged tRNAs are essential signaling molecules that can bind to and activate protein kinase GCN2. The target protein for activated GCN2 is the eukaryotic initiation factor 2α (eIF2α), which other stress-activated protein kinases can also phosphorylate. Translation initiation is reduced in all cases, decreasing the demand for amino acids. Noteworthy, GNC2 activation signals in amygdalar protein kinase C-δ (PKC-δ) neurons, as well as in macrophages, promotes WAT browning under leucine deprivation [70,71].

Several class C G-protein-coupled receptors (GPRs) have been identified as amino acid receptors [72,73]. Among others, the T1R1/T1R3 taste receptor, the calcium-sensing receptor (CaSR), and GPRC6A respond to various amino acids [73]. More recently, GPR142 has been identified as a sensor for aromatic amino acids [74]. The role of these receptors in BAT and beige thermogenesis has not been studied adequately yet; they are reported with a more systemic role in amino acid homeostasis, regulating food intake and hormone secretion, particularly in the intestine [75,76]. Nevertheless, interesting observations can be remembered. T1R2/T1R3 heterodimers, for example, promote the secretion of the glucose-regulating hormone glucagon-like peptide 1 (GLP-1) from enteroendocrine L-cells, which promote BAT and beige activation [77]. In a study utilizing live-cell imaging, researchers observed that L-phenylalanine was sensed by a specific Gq-coupled receptor known as GPR142. Activation of GPR42 by L-phenylalanine resulted in an increase in intracellular Ca^{2+} concentration; additionally, L-phenylalanine was taken up directly into the cell via Na^{+}-dependent amino acid transporter, causing membrane depolarization and enhancing GLP-1 secretion [78].

A crucial element of amino acid signaling involves the allosteric control of metabolic enzymes by amino acids or their metabolites. The most widely recognised examples of this phenomenon in mammalian cells are carbamoylphosphate synthetase (CPS1) [79] and glutamate dehydrogenase (GDH) [80]. CPS1 plays a pivotal role in regulating the entry of amino groups into the urea cycle, and it adjusts urea production to the levels of glutamate and arginine in the liver [81]. Notably, Bean et al. have found that the urea cycle links the mitochondrial dynamics protein Opa1 to white adipocyte browning [82]. GDH is regulated by leucine, either modulating glutamate levels and urea cycle activity in the liver [83] or regulating the entry of glutamate into the TCA cycle [84]. Furthermore, it was found that dietary obesity led to a significant 68% reduction of GDH activity in rat BAT [85].

An intriguing and relevant topic for future studies is the demonstration that BCAAs, through mitochondrial fatty acid synthase (FASN) II activation and BCAA catabolites (*e.g.*, isovaleryl-CoA, isobutyryl-CoA or 2-methylbutyryl-CoA), lead to accumulation of monomethyl branched-chain FAs (mmBCFAs) in cultured adipocytes [86], exported to the cytosol by adipose-specific expression of carnitine acetyltransferase (CrAT) and elongated by FASN. Brown fat displays the highest BCAA catabolic and mmBCFA synthesis fluxes. In contrast, these lipids are largely absent from liver and brain. Interestingly, the synthesis of mmBCFAs in BAT can persist even without microbiota, a significant source of mmBCFAs in animals. This suggests that BAT is essential in mmBCFA synthesis independently of microbial contributions. Feeding C57BL/6 J mice an HFD or control diet for 12 weeks followed by 3 weeks of feeding with identical diets formulated with 25% enriched [U-$^{13}C_5$,^{15}N]valine and [U-$^{13}C_6$,^{15}N]leucine, the results indicated that diet-induced obesity significantly decreases BCAA catabolic flux, with lower mmBCFA synthesis and abundance, particularly in BAT [86]. Of note, although fasting plasma glucose levels were elevated in obese animals, the authors found plasma BCAA levels unchanged, consistently with recent studies [87,88], without differences in plasma leucine and valine enrichment. Thus, predominantly synthesized in adipose depots, decreased in the context of diet-induced obesity, and increased in plasma during prolonged fasting, mmBCFAs are endogenous FAs produced by brown and white adipocytes that link mitochondrial BCAA catabolism, lipogenesis, and diet to metabolic disease. Given recent evidence that branched acyl lysine modifications also alter

mitochondrial enzyme function [89], it is possible that diversion of branched CoA intermediates to long chain FAs via CrAT is beneficial for maintaining healthy mitochondrial function.

3. Endocrine activity and metabolic fluxes of thermogenic fat

3.1. BAT/beige adipokines

An interesting question is whether the adipose amino acid profiles are implicated in the secretome activity of thermogenic fat. Like WAT, an important secretory role of BAT and beige fat has emerged. BAT transplantation has shown significant improvements in metabolic parameters in mouse models of obesity and diabetes [90–93]. Surprisingly, these studies revealed immediate improvements in glucose regulation in skeletal muscle and activation of endogenous brown and beige fat [92–94]. This suggests that the influence of 'brown/beige adipokines' extends beyond autocrine and paracrine effects, modulating systemic responses. Accordingly, the significant impact of total BAT loss on metabolic status, compared to the tissue-specific loss of *Ucp1*, strengthens the notion that BAT's ability to influence systemic energy balance is not solely reliant on non-shivering thermogenesis [95–98].

There is an ongoing debate about using the term "BAT/beige adipokine" to refer to proteins or other regulatory factors released by brown fat, with some limiting it to proteins released by the brown adipocyte cell type. However, no molecule secreted by BAT is particular to brown fat in the same way that UCP1 protein expression is, indicating a lack of complete "brown specificity." There are differing opinions regarding using the term 'brown adipokine' also based on the chemical nature of the released molecule. For instance, the debate includes whether the recognition of small non-coding RNAs (miRs) as significant signaling molecules released by BAT and targeting peripheral organs or the identification of lipidic factors secreted by BAT, which could be referred to as 'brown lipokines,' justifies the use of the term "brown adipokines". A systematic description of the currently characterized brown adipokines and, in general, bioactive factors (including brown lipokines and miRs) released by brown and beige adipocytes is beyond the scope of the present article; it has already been reviewed in outstanding papers [99–102]. Shortly, the released signaling molecules from brown adipocytes, such as nerve growth factor, neuregulin-4, and S100b protein, target sympathetic nerve endings. Additionally, vascular cells are influenced by factors like bone morphogenetic protein-8b, while immune cells are affected by molecules like C-X-C motif chemokine ligand-14. These signaling interactions contribute to the tissue remodeling associated with the adaptive recruitment of BAT in response to thermogenic stimuli. Furthermore, brown adipokines exert their effects on distant tissues and organs. For instance, fibroblast growth factor-21 (FGF-21) and myostatin, secreted by BAT, have been identified to target the heart and skeletal muscle specifically [103].

By employing proteomics technologies, researchers have analyzed the cell media of human adipocytes derived from supraclavicular brown fat and subcutaneous WAT depots in adult individuals. This analysis has identified numerous potentially secreted proteins, including hormones, growth factors, extracellular matrix proteins, and complement system proteins. Importantly, these proteins exhibit differential regulation between brown and white adipocytes. Several proteins were found exclusively in brown adipocytes, including ependymin-related protein 1 (EPDR1), which has been detected in human plasma and implicated in thermogenic determination during the process of adipogenesis [104].

In addition, untargeted metabolomics studies have revealed that apoptotic brown adipocytes release a distinct set of metabolites called the apoptotic secretome, particularly under thermoneutral conditions. Notably, this secretome is enriched with purine metabolites. The presence of these metabolites, in turn, enhances the expression of the thermogenic program in healthy adipocytes [105]. Specifically, the purine metabolite inosine has been found to promote thermogenesis in brown adipocytes. Treating mice with inosine has been shown to increase EE mediated by BAT and beige fat.

3.2. BAT/beige microRNAs

Extracellular microRNAs (miRNAs) are small noncoding RNAs that are highly conserved and regulate gene expression at the post-transcriptional level. They can suppress protein translation or mRNA degradation [106]. These miRNAs, similar to other small molecules, can be detected in the bloodstream and transported by exosomes which are small membrane vesicles that facilitate intercellular communication [107]. Studies indicate that miRNAs are crucial regulators of brown adipocyte differentiation and the browning of white adipocytes [102]. Recent evidence suggests that miR-22 expression is increased in BAT in response to cold exposure and during the differentiation of brown preadipocytes [108]. Knockout mice lacking miR-22 globally or specifically in adipose tissue exhibited whitening of BAT, reduced thermogenesis, and impaired cold tolerance. Interestingly, miR-22 activates the mTORC1 signaling pathway by directly inhibiting tuberous sclerosis 1 [108]. Another miRNA, miR-669a-5p, is upregulated in mice exposed to cold, promoting subcutaneous WAT browning. This effect was confirmed in cell culture experiments where overexpression of miR-669a-5p in adipocyte cell lines increased the expression of thermogenic genes. Conversely, cold exposure can downregulate specific miRNAs, such as miR-375 in human visceral fat, to facilitate the thermogenic programme [109].

3.3. BAT/beige extracellular vesicles

Recent advancements in nanotechnology have led to the discovery of small lipid-bound particles released by various eukaryotic cells. These particles carry a range of biological components, including fatty acids, proteins, and nucleic acids, which can be transferred from one cell to another nearby or distant cell [110–113]. Several studies have identified extracellular vesicles (EVs) secreted by adipose tissue, particularly adipocytes, as crucial players in maintaining metabolic homeostasis and communicating with the peripheral tissues [114,115]. EVs are released by all types of adipocytes (white, brown, and beige), and their production and cargo are affected in conditions such as obesity and T2D, contributing to disease progression [112,114,116–118]. In contrast, EVs derived from BAT ("batosome") have shown beneficial effects in mice fed an HFD. These batosome particles contain proteins associated with mitochondria, lipid metabolism, electron transport chain, and β-oxidation, and their administration improved glucose tolerance and hepatic steatosis [119,120]. Although human brown adipose tissue also secretes EVs, their specific role is not well understood [121]. However, it has been observed that the secretion of EVs from human BAT increases significantly during cold-induced thermogenesis, suggesting their potential involvement in this activity [114,121]. Similarly, beige adipocytes release more EVs when activated through cAMP signaling [121] and contain factors that exhibit protective effects against diabetes [122].

Furthermore, recent discoveries have revealed the ability of mitochondria to be transferred between cells, specifically from white adipocytes to macrophages, which in turn affects metabolic homeostasis. Brestoff et al. observed that mitochondria uptake by macrophages occurs in healthy WAT when macrophages undergo polarization towards an M2-like anti-inflammatory activation state [123]. The transfer of mitochondria from adipocytes to macrophages is facilitated by heparan sulfates and is reduced in cases of obesity [123]. A similar phenomenon of mitochondria transfer emerges in BAT and plays a role in regulating adaptive thermogenesis. Brown adipocytes liberate mitochondria damaged by oxidative stress through EVs to maintain their mitochondrial integrity and thermogenic capacity. Proteome analysis of EVs released by brown adipocytes reveals an abundance of extracellular exosomes and mitochondrial components. The transfer of mitochondria occurs in response to metabolic stressors experienced by brown adipocytes, such as exposure to cold or stimulation of β-adrenergic receptors.

Resident macrophages in BAT partially take up these EVs containing damaged mitochondria through phagocytosis mediated by the CD36 receptor, and lysosomes subsequently eliminate the engulfed material. Failure to effectively remove these damaged mitochondria impairs the thermogenic response of BAT to cold exposure [124].

3.4. BAT/beige metabokines

Notably, brown and beige adipose tissue have been found to secrete 3-aminoisobutyric acid (BAIBA), a metabolite generated from the breakdown of thymine. BAIBA is inversely correlated with cardiometabolic risk factors and has been shown to induce the browning of WAT and enhance hepatic (insert the symbol for beta)-oxidation [125]. In further exploration of metabolites that could promote browning, the same scientists conducted metabolic profiling of media conditioned by browning adipocytes. By treating differentiating brown fat cells with cyclic AMP (forskolin) and PPARδ (GW0742) agonist in culture, they identified 3-methyl-2-oxovaleric acid (MOVA), 5-oxoproline (5-OP), and β-hydroxyisobutyric acid (BHIBA) as a unique group of small molecule "metabokines" synthesized in brown and beige fat and secreted via monocarboxylate transporters. MOVA, BHIBA, and β-hydroxyisovaleric acid (BHIVA) are monocarboxylic acids produced during the BCAA catabolism. Notably, β-aminoisobutyric acid, a catabolite of valine, functions as a myokine stimulated by exercise, signaling to induce WAT browning and hepatic fatty acid oxidation [125]. BHIBA, also derived from valine metabolism, facilitates communication between skeletal muscle and the endothelium in a peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α)-dependent manner, increasing fatty acid uptake [126]. These metabolites have demonstrated the ability to reduce adiposity, enhance EE, and improve glucose and insulin homeostasis in mouse models of obesity and diabetes. These effects complement the actions of BATokine proteins and lipokines.

More recently, Park et al. developed an arteriovenous (AV) metabolomics method to quantify tissue-specific metabolic flux during thermogenesis in mice, focusing on BAT and skeletal muscle [127]. They collected systemic arterial blood from the left ventricle, while venous blood from Sulzer's vein (for BAT) and femoral vein (for the leg) was obtained. The net uptake and release of metabolites by BAT and the leg were determined by analyzing the AV gradients of individual metabolites. Cold adaptation was compared to thermoneutrality as the standard. It was observed that during chronic cold exposure (gradual drop from 30 °C to 6 °C over four weeks), BAT exhibited significant uptake of various fuel metabolites such as glucose, lactate, 3-hydroxybutyrate (a ketone body), medium-chain fatty acids, and long-chain non-esterified fatty acids. In cold adaptation, BAT also showed a marked increase in nitrogen uptake through the net consumption of amino acids, except glutamine. Isotope tracing and functional studies indicated concurrent glutamine catabolism and synthesis via glutamine synthetase, which prevents ammonia buildup and enhances fuel oxidation [127]. Regarding carbon efflux, cold-adapted BAT released succinate as the only TCA cycle intermediate. The selective release of succinate is supposed to be related to its unique adrenergic-independent accumulation in BAT and its role in generating reactive oxygen species. Alternatively, specific BAT cells might export succinate in response to hypoxia [128], considering that BAT extracts almost all the oxygen from the blood [2129,130].

A direct association between different brown or beige secretomes – including BATokines, miRNAs, EVs, and metabokines – and changes in amino acid profile in adipocytes or adipose tissues is an area for future investigation.

4. Amino acid quantity and combination in foods modulate animal and human metabolic phenotypes

Since the 1960 s, high-protein diets (with more than 15% protein as a source of energy) have been promoted as a way to lose weight and prevent obesity and its metabolic complications [131,132]. However, growing evidence of increased cardiovascular risk has raised doubts about these diets' long-term safety [133]. As a result, the demonstration that diets low in protein or low in essential amino acids (EAAs) like leucine, methionine, or tryptophan were linked to improved energy balance and decreased overweight or obesity in humans and mice [134, 135] generated a lot of excitement. In contrast, several studies have demonstrated that the central or peripheral administration of a single amino acid, such as glycine, leucine, or tryptophan, positively impacts body weight and/or energy metabolism [136,137]. However, not everyone agreed with these findings [138].

We conducted in-depth analyses of the metabolic effects caused by the acute and chronic consumption of two customized diets in normal-weight and obese mice to investigate these contradictory results. In particular, the protein content (i.e., casein) of the SFA diet (10% fat) and high-fat eating routine (HFD, 60% fat) was substituted with a unique formula made by EAAs (SFA-EAA and HFD-EAA) (for nutrient compositions see below). We previously demonstrated that when taken as a dietary supplement with water, the EAA mixture improved health, particularly in older adult mice, and promoted mitochondrial biogenesis in middle-aged mice's skeletal and cardiac muscles [139,140]. In particular, the EAA mixture prevented oxidative stress in metabolically active cells by drinking supplements [140–142], improving muscle and cognitive performance in various animal models and humans [143,144]. Furthermore, a proper restoration was seen in the gut microbiota of aging mice with the same EAA formula [145]. This observation appears relevant because intestinal microbiota may also influence energy balance through diet composition (see the paragraph below titled "The role of microbiota in the modulation of amino acid metabolism and thermogenesis activation").

To maximise the concentrations of EAA mixture, we conceived the SFA-EAA and HFD-EAA diets, and we investigated their metabolic impacts in obese mice models comparing the effects with regular chow diet, the respective control diet (SFA or HFD), and with an additional control diet in which casein was substituted with the amino acids designed on casein profile. Chronic consumption of the EAA-substituted diets prevented and reverted obesity and T2D development, extending the life span of male mice. Accordingly, EAA-fed mice owned improved glucose homeostasis and insulin sensitivity and ameliorated liver functions. Of note, after a few weeks of feeding (acute regimen), the EAA-substituted diets stimulated BAT thermogenic program and shaped microbiota towards a healthy phenotype [87]. Notably, we found that the SFA-EAA diet reduced tumour growth in vivo, promoting endoplasmic reticulum stress and inhibiting mTOR activity in cancer cells [87]. Additionally, the SFA-EAA diet prevented and treated heart failure in a left ventricle pressure overload model in mice, renormalizing BCAA oxidation in cardiac tissue, which is suppressed in the failing heart [146].

5. The role of microbiota on amino acid metabolism and thermogenesis

According to growing evidence, there are connections between the human microbiome's composition and metabolic health [147,148]. However, the relationship between a human metabolic phenotype, gut microbiome, and diet is inextricably intertwined, multifactorial, and multidirectional. Meta-omic approaches (untargeted, high-throughput

genomic and molecular approaches applied to microbial communities) have expanded beyond merely profiling the high-level taxonomic composition of the microbiome to characterizing microbiome members at the resolution of single genomes, surveying microbial gene expression, and evaluating the metabolites that are produced by these microbes increasing the ability to disentangle this intricacy [149–151]. More than host genetics [152], diet also influences the composition and characteristics of the gut microbiome [153], making it a stronger predictor of metabolic disorders. Microbes in the gut have distinct dietary preferences [154]. While some bacterial species, such as those in the *Firmicutes* phylum, utilize all three primary sources of nutrients in the intestine—carbs, proteins, and lipids—many others are specialized toward particular nutrients. *Bifidobacterium* species primarily metabolize carbohydrates, whereas *Alistipes* species metabolize proteins [155].

The microbiome is influenced over time by dietary habits [156–158]; it has also been demonstrated that short-term dietary interventions can quickly alter its composition [159]. For instance, EAA-derived short--chain fatty acids alter the overall lipid balance and glucose metabolism and consuming amino acids increases the relative abundance of *Bacteroidetes* [156,160]. In addition, mice's adaptive thermogenesis can be triggered by the gut microbiota, which controls adiposity [161]. Amino acid metabolism is manipulated and synthesized by intestinal microbiota, which has implications for systemic physiology; additionally, the microbiota landscape is altered by diet, which substantially affects the metabolism of amino acids [139]. We noticed some fascinating contrasts concerning microbiota organization. *Allobaculum* and *Sutterella* were more abundant in the intestines of mice fed the SFA-EAA diet than on the SFA or SFA-CAA diets. This may play some role in the inverse correlation between body weight and insulin resistance, which aligns with previous research [162]. Multiple dietary interventions, such as prebiotics, probiotics, and berberine – which ameliorate insulin resistance and adipose inflammation – promote the relative abundance of both *Allobaculum* and *Sutterella*. At the same time, several studies have demonstrated that HFD reduces the relative abundance of both taxa in the gut of mice [163, 164].

Once more, mounting evidence suggests intestinal microbiota can stimulate brown fat thermogenesis in mice [162,165]. It is necessary to conduct additional research to establish metabolic connections between the activation of the BAT by our diets and gut bacteria like *Allobaculum* and *Sutterella*. In this specific context, after showing that plasma BCAA and BCKA levels correlated with body weight in humans and mice and this is connected to BCAA catabolic defects in BAT causally associated with obesity, Yoshida et al. demonstrated that treatment with *Bacteroides dorei* and *Bacteroides vulgatus*, the dominant species within Bacteroides spp., improves BAT BCAA catabolism and inhibits obesity in dietary obese mice [166]. Beyond glucose intolerance amelioration, *Bacteroides*-gavaged mice had significantly lower concentrations of BCAA, BCKA, isobutyryl-CoA, and acetyl-CoA than control mice. Mice gavaged with *Bacteroides* also had substantially smaller adipocytes in their inguinal fat mass. Similarly, the epididymal fat tissue had smaller adipocytes, less macrophage accumulation, and less fibrosis. In addition, the BAT weight of *Bacteroides*-treated mice was significantly lower than that of control mice, and UCP1 expression was also higher. According to these findings, *Bacteroides* spp. may significantly regulate BAT's BCAA catabolism, and the gut microbiota is an essential environmental factor controlling this process and preventing obesity (Fig. 2).

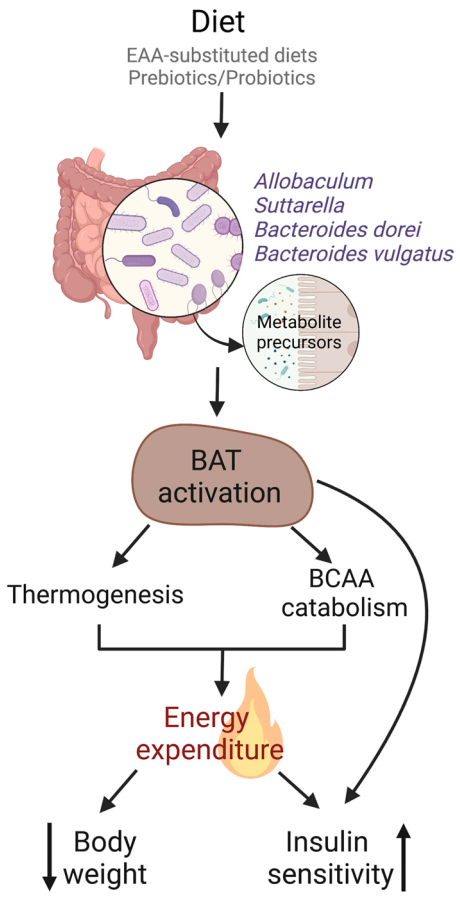

Fig. 2. Shaping intestinal microbiota influences BAT thermogenesis and whole energy metabolism. The metabolic disease (*e.g.*, obesity and T2D) development has been associated with alterations of the gut microbiota composition, suggesting a role of gut bacteria in the regulation of adiposity and glucose homeostasis. Modifying the feeding habits (*e.g.*, consumption of foods enriched in carbohydrates, fat, or protein, as well as prebiotics or probiotics and essential amino acid supplement) shapes the gut microbiota, increasing the abundance of specific bacteria that produce metabolic intermediates (i.e., short-chain fatty acids) able to promote a healthy metabolic state. Of note, the abundance of certain bacteria (*e.g.*, *Bacteroides*, *Suttarella*, *Allobaculum*) was associated with activating the BAT adaptive thermogenic program and increasing the BCAA catabolism. Created using BioRender.com.

6. Amino acid landscape in thermogenic fat as a target of the novel metabolic drugs

The aminograms (i.e., the tissue amino acid profiles) of activated BAT, such as in mice exposed to a cold environment or calorie restriction, or treated with β_3-AR agonists or amino acid-substituted diet, share many similarities. For example, arginine, isoleucine, leucine, proline, threonine, and valine are statistically higher in SFA-EAA– than in control diet–fed mice, while, on the contrary, glycine, lysine, and serine are lower [87] (Fig. 3). Similarly, raised levels of glutamine, proline, tryptophan, and phenylalanine content in addition to the BCAAs in BAT have also been reported in response to cold exposure [26,55,167] (Fig. 3). These results suggest causal thermogenic amino-acid combinations. Although not conclusively demonstrated in all studies, BCAA supplementation ameliorates some metabolic dysfunction caused by obesity or diabetes without impairing glucose metabolism [168,169]. As a substrate of nitric oxide synthase, arginine produces nitric oxide (NO) for signaling purposes, including mitochondrial biogenesis and thermogenic program in BAT [170] (Fig. 3). Increasing evidence has shown that dietary supplementation of arginine can effectively improve BAT thermogenesis via the mTOR signaling pathway [171], with reduced obesity and diabetes, in addition to ameliorated obesity-linked dyslipidemia and high blood pressure in mammals, including humans [172]. Similarly, proline supplementation improves NO bioavailability and counteracts blood pressure [173]. By contrast, the SFA-EAA diet reduced the non-essential amino acid (NEAA) glycine levels in BAT compared with the SFA-CAA diet, and accordingly, the glycine precursor threonine was increased. Of note, neuronal glycine has been found to inhibit sympathetic activation of BAT [174] (Fig. 3). Although circulating glycine is low in obese subjects, and its supplementation was proposed to treat

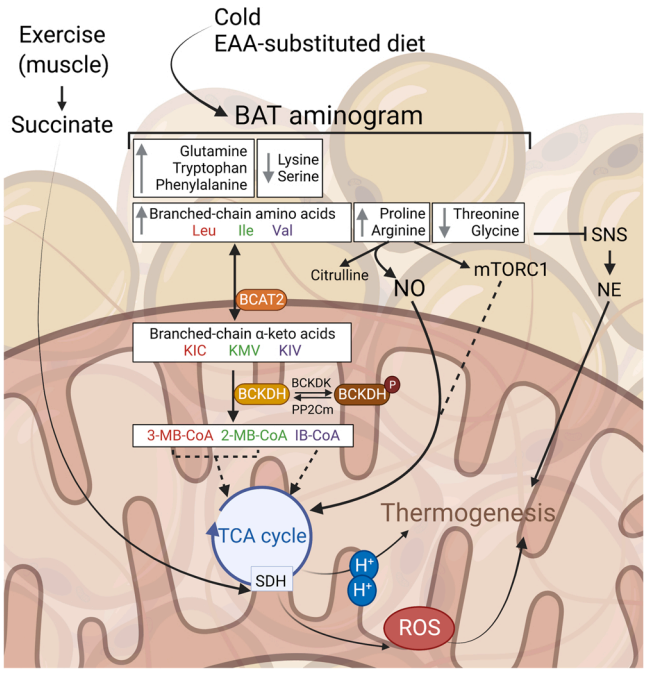

Fig. 3. Amino acids and TCA cycle intermediates as thermogenic activators. New insight demonstrated that BAT owns a peculiar amino acid profile (also named aminogram) in the presence of thermogenic stimuli (e.g., cold exposure or EAA-substituted diets). Specific treatments modulate the amino acid levels in BAT similarly during the thermogenic program activation. Activated BAT exhibits increased BCAA levels accompanied by high BCAA catabolism (i.e., BCAA oxidation), which plays a relevant role in adaptive thermogenesis. Activating the crucial enzymes of BCAA oxidation (BCAT2, BCKDH, and PP2Cm) results in the production of acetyl-CoA and succinyl-CoA (i.e., TCA cycle intermediates), stimulating the mitochondrial function and, in turn, thermogenic BAT capacity. The BCKDH complex is inactive when phosphorylated by BCKDK and active when dephosphorylated by PP2Cm. The increase in arginine levels leads to thermogenic activation: arginine is the specific substrate of the endothelial nitric oxide synthase (eNOS), which enzymatically transforms the amino acid in citrulline with NO production. Increased levels of NO, a free radical, promote mitochondrial biogenesis and brown fat thermogenesis. Additionally, arginine directly stimulates the mTOR signalling pathway, a nutrient-responsive sensor. Several amino acids (e.g., arginine and leucine), exercise, and growth factors are crucial modulators of multiple metabolic pathways, including mitochondrial biogenesis and function, acting through mTORC1. Moreover, decreasing glycine levels and its precursor threonine in BAT activates the thermogenic program, given that neuronal glycine release inhibits the sympathetic nervous system (SNS) activity and norepinephrine (NE) release. Alternatively, the selective BAT accumulation of succinate - normally produced by skeletal muscle contraction and transported by blood flow across the organs - can be a relevant source of thermogenic ROS in brown and beige fat [44]. This activity of succinate is independent of the lipolytic cascade and relies on its oxidation by mitochondrial succinate dehydrogenase (SDH) and consequent ROS production. Thermogenic adipocytes have the capacity to sequester succinate from the circulation, indicating that this regulation can be manipulated through the increase of systemic succinate levels (for example, with dietary succinate supplements). Ile, isoleucine; Leu, leucine; Val, valine; BCAT2, mitochondrial BCAA aminotransferase; BCKDH, branched-chain α-ketoacid dehydrogenase complex; BCKDK, branched chain ketoacid dehydrogenase kinase; PP2Cm, protein phosphatase 2Cm; KIC, ketoisocaproic acid; KMV, α-keto-β-methylvaleric acid; KIV, α-ketoisovaleric acid; 3-MB-CoA, 3-methylcrotonyl-CoA; 2-MB-CoA, 2-methyl-3-hydroxy-butyryl-CoA; IB-CoA, 3-hydroxy-isobutyryl-CoA; mTORC1, mechanistic target of rapamycin complex 1; NO, nitric oxide; PP2Cm, protein phosphatase 2Cm. Created using BioRender.com.

obesity [175], no clear role has been found in BAT. Also for serine, which was similarly reduced in SFA-EAA mice, further studies are needed to confirm and extend our findings.

7. Autophagy roles in thermogenesis

7.1. Autophagy: mechanisms and regulation

The roles of autophagy in thermogenesis and therapeutic potential are active research fields; in addition, autophagy is critical for amino acid response and implicated in metabolic diseases. Outstanding review articles have summarized the mechanisms and regulation of autophagy, as well as its relevance in adipose biology, BAT/beige thermogenesis, and metabolic disorders [176–180]. Autophagy is a cellular process that degrades cytoplasmic substrates by delivering them to lysosomes. This catabolic process produces molecules that can be recycled and used as energy sources or for the synthesis of new cellular components [181]. Basal autophagy occurs in almost all cell types and serves as a mechanism to maintain normal cellular functions under non-stressful conditions. It contributes to cellular homeostasis by degrading unnecessary or damaged proteins and organelles as a quality control mechanism. Autophagy can also be induced in response to various cellular stresses or as a remodeling mechanism to modify the composition of intracellular proteins and organelles, thereby contributing to cellular plasticity [182]. Among the different types of cellular stresses that can modulate autophagy (such as oxidative stress, hypoxia, endoplasmic reticulum stress, and starvation), those related to metabolic stress and cellular plasticity are particularly relevant in adipocytes (Fig. 4). Autophagy is classified into three types based on cargo recognition and degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [183]. Microautophagy and CMA rely on a recognition sequence known as the KFERQ motif in their substrates. In microautophagy, the lysosome directly engulfs and degrades cytoplasmic organelles. In CMA, chaperones select the cargo and facilitate its degradation within the lysosome [184].

CMA plays a regulatory role in various cellular processes, including adipocyte differentiation, by remodeling the proteome and timely degrading key regulatory signaling proteins and transcription factors involved in proliferation, energy adaptation, and signaling changes required for adipogenesis [185]. Macroautophagy, often referred to simply as "autophagy," involves the formation of a double-membrane vesicle called an autophagosome around the targeted cytoplasmic material. This autophagosome can contain proteins, cytoplasmic inclusions (such as glycogen), organelles, or intracellular pathogens. Macroautophagy begins with the formation of the autophagosome (nucleation), followed by elongation of the double membrane, transport through the cytoplasm, and fusion with the lysosome (Fig. 4).

The most extensively studied signaling pathways that regulate autophagy in mammals are those triggered by growth factors and cellular nutritional status. These pathways converge at the functions of mTOR complex 1 (mTORC1) and AMP-activated protein kinase (AMPK), which critically sense the cell's energy state and control the switch between protein synthesis and autophagy. Under nutrient-rich conditions, mTORC1 is active, promoting protein synthesis and inhibiting autophagy. Conversely, under low-energy conditions, AMPK is activated, leading to the induction of autophagy by inhibiting the actions of mTORC1. Recent research has elucidated the mechanisms by which mTORC1 senses amino acid concentrations in both the cytosol and lysosomes (partly summarized in Fig. 4). For instance, during acute leucine starvation, the cytosolic leucine sensor known as Sestrin2 binds and inhibits GATOR2, preventing the recruitment of mTORC1 to lysosomes [191]. Upon refeeding and restoration of leucine levels, leucine binds to a specific pocket on Sestrin2, causing its dissociation from GATOR2 and relieving the inhibition of mTORC1 [191,195]. Sestrins are transcriptionally upregulated by ATF4 – a transcription factor that upregulates genes involved in amino acid import, glutathione

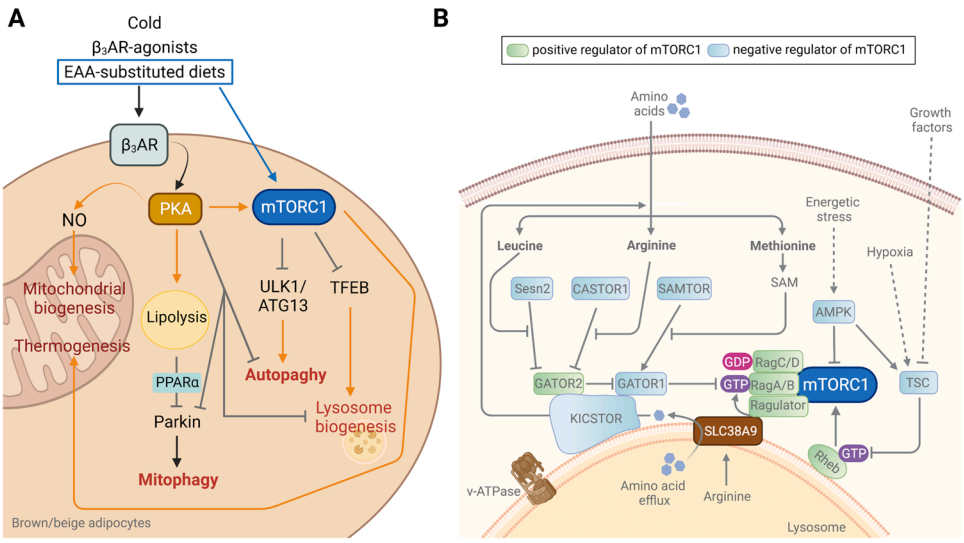

Fig. 4. Autophagy and mTORC1 signaling pathway in thermogenic fat. **(A)** Thermogenesis activation is paralleled by repression in the autophagic activity in mature brown/beige adipocytes. After exposure to thermogenic stimuli [e.g., cold, β_3-adrenergic receptor (β_3-AR) agonists, essential amino acid (EAA)-substituted diets], the protein kinase A (PKA) is activated by the β_3-AR signalling cascade, and induces lipolysis, thermogenesis, and mitochondrial biogenesis. In parallel, PKA directly inhibits the expression of autophagic and lysosomal biogenesis genes. The mechanisms responsible for the transcriptional repression of PKA-mediated autophagy in brown and beige adipocytes are substantially unclear. Some evidences demonstrate that melanocyte-inducing transcription factor (MITF) and forkhead box O3 (FOXO3) as being autophagy-related transcription factors that are downregulated by the action of PKA in beige adipocytes, together with its autophagic- and lysosomal-related targets [186]. The PKA-mediated autophagic repression in brown/beige adipocytes is also likely due to indirect regulation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway activation. The β_3-AR agonists trigger PKA to phosphorylate and activate mTORC1 principal components (mTOR and Raptor); mTORC1 represses autophagy through the inhibitory phosphorylation of unc-51-like autophagy-activating kinase 1 (ULK1) and autophagy-related 13 (ATG13), two critical early effectors in the induction of autophagy, driving to the formation of the autophagosome [187]. The mTORC1 pathway is also involved in lysosome biogenesis through phosphorylation of the transcription factor EB (TFEB), a regulator of the expression of lysosomal genes, thereby inhibiting the lysosomal degradation activity [187]. PKA also downregulates mitophagy by inhibiting the translocation of Parkin to depolarized mitochondria. The PKA-mediated lipolysis induction inhibits the Parkin gene transcription through the action of peroxisome proliferator-activated receptor alpha (PPARα). **(B)** Amino acids repress autophagy via mTORC1 activation. Amino acids directly activate mTORC1 machinery in BAT/beige adipocytes, modulating thermogenesis also independently of the β_3-AR stimulation [87]. Leucine, arginine, and S-adenosylmethionine (SAM, a methionine metabolic byproduct) are sensed by cytosolic and lysosomal sensors involved in the Rag-GTPase activation, which is the essential competent to recruit mTORC1 in the presence of amino acids [188]. The Rag-GTPase is a heterodimer anchored to the lysosome by the Ragulator complex and configured such that RagA or RagB is bound to RagC or RagD. When Rag-GTPase is activated, RagA/B is bound to GTP and RagC/D to GDP. In the presence of amino acids, Raptor – a subunit owned by mTORC1 – links the activated RagGTPase, and this interaction stimulates the recruitment of mTORC1 from the cytosol to the lysosome. Once localized to the lysosomal surface, the mTORC1 kinase activity can be stimulated by the lysosomal GTPase Rheb in the active form (i.e., the GTP-bound state). Rheb activation is also promoted by growth factors and opposed by energetic stress or hypoxia via the modulation of tuberous sclerosis complex (TSC) and AMP-activated protein kinase (AMPK) signaling pathway [187]. The most direct regulator of Rag status is GAP activity towards the Rags 1 (GATOR1) complex, which transmits cytosolic amino acid signals to RagGTPase [189]. When the cytosolic amino acid levels are low, GATOR1 inhibits the mTORC1 pathway by hydrolysis of GTP bound to RagA/B. In turn, GATOR1 activity is regulated by the KISTOR complex, which anchors GATOR1 to the lysosome and is necessary for cellular sensitivity to amino acid deprivation [190], and GATOR2 complex that antagonizes GATOR1 function and acts as potent stimulator of mTORC1 [187]. Two crucial amino acid sensors, Sestrin 2 (Sesn2) and cytosolic arginine sensor for mTORC1 subunit 1 (CASTOR1), relay the cytosolic availability of leucine and arginine to the mTORC1 pathway through the interaction with GATOR2. Sesn2 binds and inhibits GATOR2 during leucine starvation, preventing the mTORC1 lysosomal recruitment. Restoring leucine levels allows binding of the amino acid with Sesn2, which dissociates from GATOR2 and activates mTORC1 [191]. Likewise, CASTOR1 inhibits GATOR2 when levels of arginine are low and dissociates from the complex when arginine is bound. Another arginine sensor is the SLC38A9 transporter on the lysosomal membrane, which detects amino acid levels inside the lysosomal lumen and allows the amino acid efflux outside the organelle [192]. The SLC38A9 N-terminus domain interacts with Ragulator to maintain the RagGTPase into the active state by promoting GTP loading of RagA/B [193]. Similarly, the lysosomal v-ATPase, which maintains the pH gradient of the lysosome, communicates with the Rag–Ragulator complex to influence the GTP-loading state of the RagGTPase [194]. Finally, it has been found that mTORC1 also responds to amino acid metabolites, such as SAM, derived from methionine. S-adenosylmethionine sensor (SAMTOR) negatively regulates mTORC1 by binding GATOR1 and KISTOR in the absence of methionine or SAM. Rescuing the cytosolic SAM level promotes the SAMTOR dissociation from GATOR1, stimulating the mTORC1 activity. Created using BioRender.com.

biosynthesis, and the antioxidative stress response [196,197] – and the endoplasmic reticulum unfolded protein response [198]. Sestrin overexpression alone is sufficient to suppress mTORC1 signalling in vitro [199,200]. Increased levels of amino acids such as leucine, arginine, and methionine directly enable the interaction of mTORC1 with the ULK1 complex, resulting in phosphorylation of ULK1 at inhibitory sites and blocking the early steps of autophagy initiation [201]. Conversely, when ATP levels are low, AMPK phosphorylates ULK1 at activating sites, disrupting its inhibitory interaction with mTORC1. Additionally, mTORC1 phosphorylates transcription factor EB (TFEB), the primary transcription factor responsible for inducing the expression of autophagy-related genes and lysosomal biogenesis [202]. Phosphorylating TFEB hinders its movement into the nucleus, resulting in the suppression of its transcriptional activity [203].

7.2. *Autophagy and thermogenesis in brown/beige fat*

While the involvement of autophagy in the differentiation process of white-versus-brown/beige adipocytes has been established [178], our focus lies on understanding the role of autophagy in thermogenesis within BAT and beige adipose tissue. It has been observed that thermogenic activation is accompanied by a decrease in the autophagic degradation activity in BAT [186,204,205]. The regulation of both processes intersects at PKA, which activates the thermogenic program in brown adipocytes while inhibiting autophagy (Fig. 4).

In a recent study, it was discovered that the stimulation of β_3-adrenergic receptors in brown and beige adipocytes leads to the activation of PKA, which phosphorylates and activates critical components of mTORC1, namely mTOR and Raptor [206]. Notably, the activation of mTORC1 in response to cold exposure appears to be crucial for the proper initiation of the thermogenic program during short-term cold exposure and the expansion of brown and beige adipose tissues during

long-term cold adaptation [42,206–208]. Since both PKA and mTORC1 are required for cold-induced thermogenesis and are known to inhibit autophagy, it is plausible that the effects of the PKA-mTORC1 axis on enhancing thermogenesis involve the parallel suppression of autophagy (Fig. 4).

Mitophagy, a process of macroautophagy that facilitates the intracellular degradation of mitochondria, plays a critical role in eliminating damaged or dysfunctional mitochondria and maintaining cellular homeostasis. The most well-known molecular mechanism involved in mitophagy is the PTEN-induced putative kinase 1 (PINK1)/Parkin-mediated pathway, which is activated in response to a decline in mitochondrial membrane potential, typically indicating mitochondrial dysfunction. Regulating the PINK1/Parkin system is essential for preserving the mitochondrial pool in thermogenically activated brown and beige adipocytes. Similar to other autophagic proteins, Parkin is induced during the differentiation of brown and beige adipocytes, coinciding with the increase in mitochondrial content [204,209,210]. In contrast, PINK1 is primarily regulated at the post-transcriptional level since it is rapidly degraded by mitochondrial proteases in healthy mitochondria, resulting in generally low levels of PINK1. However, when thermogenesis is acutely induced by cold, PINK1 is stabilized at the surface of depolarized but healthy mitochondria in BAT [204]. On the other hand, Parkin is significantly downregulated in response to thermogenic stimuli. Its transcription is strongly repressed through mechanisms mediated by peroxisome proliferator-activated receptor-alpha (PPARα), and its translocation is impaired by the activation of PKA during thermogenesis. This regulatory process enables active mitochondria to avoid degradation even when physiological depolarization stabilizes PINK1. It is important to note that mice lacking PINK1 and exposed to cold exhibit mitochondrial defects in BAT [209], whereas cold-exposed mice lacking *Parkin* do not display such defects [204,209,210]. This suggests that there might be redundant downstream mechanisms of PINK1 involved in regulating mitophagy, as described by Lazarou et al. [211], which contribute to maintaining mitochondrial quality control in the absence of Parkin.

Brown and beige adipocytes substantially reorganize the mitochondrial network upon adrenergic stimulation. This reorganization results in mitochondrial fragmentation, which, in turn, enhances the oxidative capacity of these adipocytes [212,213]. The fragmentation of mitochondria observed may indicate an increase in the mitochondrial surface area, facilitating the efficient importation of oxidative substrates. These substrates can originate from the hydrolysis of lipid droplets or be taken up by the cells from the bloodstream, including triglycerides and glucose.

7.3. Autophagy in human brown/beige fat and its role in obesity and lipodystrophy

In addition to its impact on lipid metabolism and maintaining mitochondrial quality during prolonged exposure to cold, autophagy may also influence various other cellular processes in BAT. Cold acclimation has been observed to promote hyperplasia in BAT, characterized by significant proliferation of preadipocytes and vascular endothelial cells [214–216]. In this context, it has been discovered that autophagy plays a role in regulating both the differentiation of brown adipocytes [217,218] and angiogenesis [219,220].

Autophagy has been reported to be increased in adipocytes of individuals with obesity [221–223]. Consequently, the therapeutic modulation of autophagy has been proposed as a potential pharmacological strategy to prevent or reverse metabolic disorders associated with obesity. However, conflicting findings have been observed regarding the extent of autophagy enhancement in adipocytes of obese patients. Dugail and collaborators found that adipocytes isolated from individuals with obesity exhibited lower levels of autophagic activity than those isolated from lean subjects [224].

Autophagic changes in adipose tissues also play a role in non-obesity-related conditions, including lipodystrophic syndromes. A recent study utilized adipocyte precursor cells from patients with Familial partial lipodystrophy type 2 (FPLD2), a laminopathic disorder, and found that dysregulation of autophagy during adipogenesis (initial activation followed by a blockade of the autophagic process) could have varying effects on adipogenesis depending on the specific fat depot. This dysregulation led to subcutaneous fat loss and fat accumulation in the neck region [225].

Numerous questions still require clarification in this field: (i) Are the alterations in autophagic activity observed in adipose tissues of obese individuals the cause, consequence, or both of adipose tissue disturbances? (ii) Are there any associations between circulating amino acids and autophagy in BAT or beige fat in the context of obesity? (iii) Can anti-obesity drugs modulate autophagy in thermogenic fat through amino acid metabolism?

8. A dual agonist that induces a thermogenic-like amino acid signature in BAT: the case of tirzepatide

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor-based multi-receptor agonists are a new generation of therapy for metabolic diseases (Fig. 5). Compared to treatment with selective GLP-1 receptor (GLP-1R) agonists, tirzepatide, a dual GIP and GLP-1 receptor agonist, has demonstrated superior weight loss in patients with T2D [226,227]. The ability of multi-receptor agonists to intervene at complementary regulatory pathways is a desirable property crucial to fighting metabolic diseases' complexity. Notably, the weight-independent glycemic benefits of tirzepatide in obese and T2D mice and humans are accompanied by a decrease in circulating BCAAs and BCKAs [228,229]. Branched-chain amino acid aminotransferase 2 (BCAT2) and branched-chain alpha-keto acid dehydrogenase (BCKDH), which are mitochondrially located enzymes that catalyze the first and second steps of BCAA catabolism, respectively, in BAT are associated with these whole-body effects [230] (Fig. 3). This leads to the hypothesis that tirzepatide reaches its beneficial advantages, to some extent, by increasing the oxidation of BCAAs in the BAT mitochondria for thermogenesis with systemic BCAA clearance.

Samms et al. used stable-isotope tracer studies and metabolomic analyses to study obese insulin-resistant mice after chronic tirzepatide treatment. Despite a similar reduction in body weight between the two groups, they have recently discovered that long-term (14 days) tirzepatide treatment had a distinct impact on amino acid levels in BAT when compared to a pair-feeding (PF) group [231]. BCAA levels in BAT were significantly higher than those in control animals, as expected, but not in PF animals. In addition, relative to the PF or vehicle-treated group, the drug increased the levels of several additional amino acid species, including arginine, citrulline, histidine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, and tyrosine. This amino acid signature in BAT is similar to the amino acid profile in activated BAT following cold exposure and the SFA-EAA diet [26,87,232] (Figs. 3 and 5). These results suggest that tirzepatide induces a thermogenic-like amino acid profile in BAT. This effect may contribute to the benefits of tirzepatide treatment in obese mice, including increased metabolic rate, weight loss, and ameliorated insulin resistance.

However, deleting *Bcat2* in WAT in mice results in resistance to HFD-induced obesity, primarily due to enhanced browning and thermogenesis in subcutaneous WAT [233]. Depletion of acetyl-CoA derived from BCKAs strongly promotes the browning of WAT and EE. Conversely, supplementation of BCKAs reinstates dietary obesity in *Bcat2* knockout mice. While the GLP-1R agonist liraglutide, known for its anti-diabetic and anti-obesity effects, induces the development of beige fat and enhances mitochondrial function in diet-induced obese rodents through miR-27 [234–236], it remains to be investigated whether its metabolic benefits are linked to amino acid metabolism in browning WAT. Notably, Park et al. demonstrated that liraglutide restored plasma BCAA levels reduced by HFD [237]. The authors also suggested that examining

the metabolic profiles of various essential and non-essential amino acids, which serve as substrates for the TCA cycle, urea cycle, glycolysis, or gluconeogenesis, could enhance our understanding of the role of amino acids in liraglutide therapy. However, the metabolic profiles of BAT and beige fat were not investigated, and it is important to consider the possibility of indirect modulation of amino acid-dependent adipose thermogenesis by liraglutide and other GLP-1R agonists through the brain or other tissues. Therefore, definitive conclusions cannot be drawn at this stage regarding the involvement of amino acid metabolism and/or GLP-1 agonism in WAT browning and beige fat thermogenesis.

9. Glucagon receptor-targeted multi-agonists: amino acid metabolism and EE

There are currently several potential partners for GLP-1 and GIP receptor agonists under investigation, but glucagon receptor (GCGR) agonists have recently emerged as additional candidates for further research. A monomolecular co-agonist of GLP-1R and GCGR was the first to emerge, combining GLP-1 anorectic effects with GCG's ability to increase EE [243–245]. It is meaningful to mention that the insulin-triggering properties of GLP-1 must neutralize the diabetes-causing effects of GCGR stimulation, which usually leads to the creation of co-agonists with more potency for GLP-1R compared to GCGR potency, with up to a ten-fold reduction in relative GCGR potency due to molecular design [246–248].

Shortly after the purification and crystallization of GCG [249,250], various studies showed that it could boost metabolic rate, promote high oxygen consumption, and increase EE in both humans and rodents (for references see [251]). It is worth emphasizing that EE was seen to increase in rats in a fasted state without a corresponding increase in blood glucose levels [252]. GCG has been found to increase oxygen consumption, temperature, and blood flow toward BAT in rodents, suggesting increased metabolic activity in this tissue [253–255]. This effect becomes more pronounced during cold acclimatization and is reduced with thermoneutrality [254–256]. Recent studies have highlighted that GCG plays a vital role in adaptive thermogenesis in BAT by expanding thermogenic machinery, particularly UCP1 and mitochondrial differentiation markers [257,258]. Additionally, GCG increases BAT thermogenic capacity by driving mitochondrial biogenesis, stimulating metabolic gene expression, and promoting de novo adipogenesis [258–260]. The thermogenic potential of beige adipose tissue may still be leveraged even though GCG does not require BAT per se. It is indeed found that the GCGR is expressed in WAT, where its stimulation triggers oxygen consumption and the expression of thermogenic genes (resulting in WAT browning) [260]. Nevertheless, even with these discoveries, more recent investigations that utilized transgenic models suggest that the signaling mechanism of GCG to the standard UCP1 thermogenic pathway in BAT tissue is not obligatory for the entire-body expense of

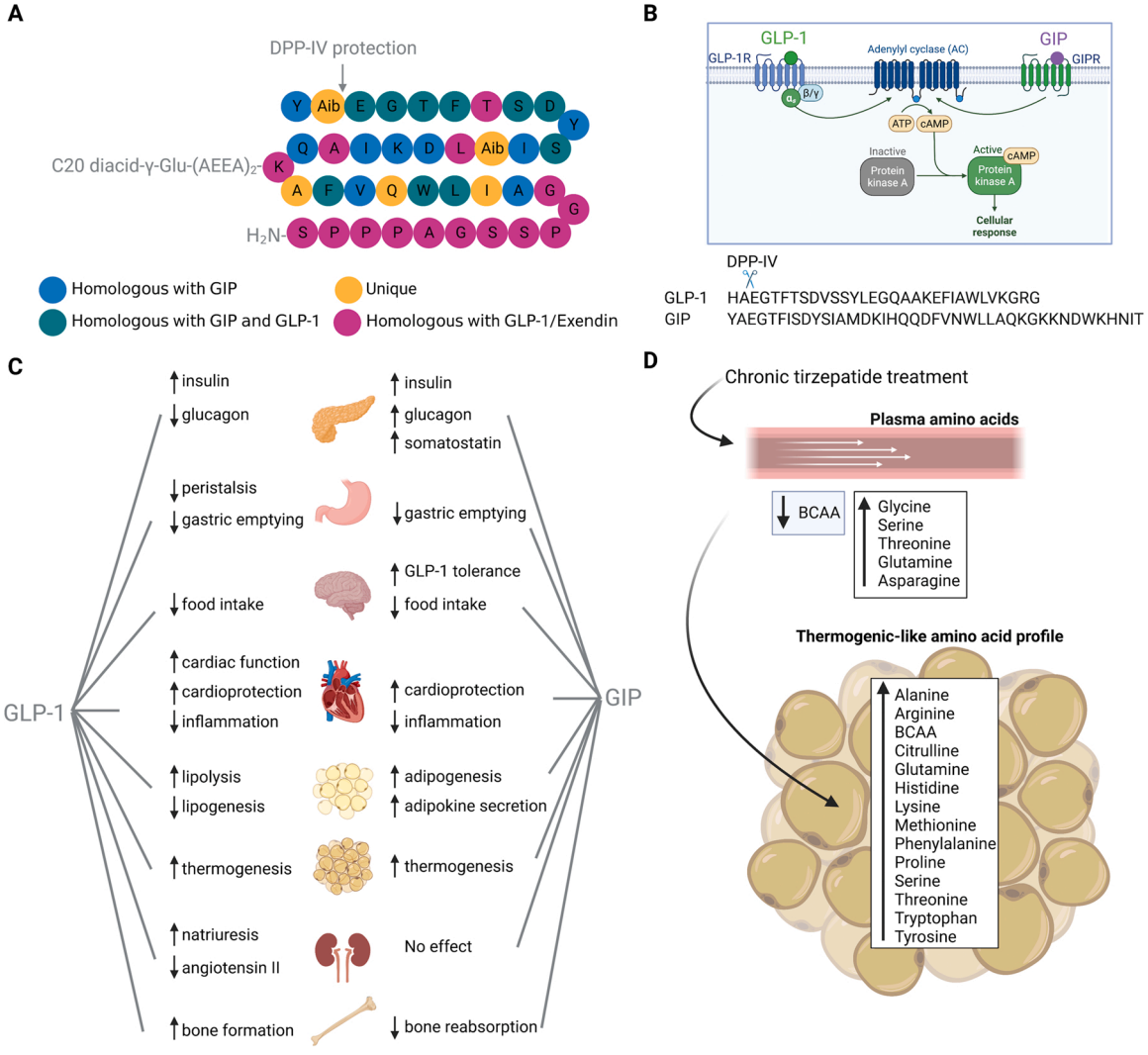

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Fig. 5. The dual agonist tirzepatide induces a thermogenic-like amino acid signature in BAT. (**A**) Tirzepatide is a GIP analogue that activates the GLP-1 and GIP receptors, improving blood sugar control. Tirzepatide (molecular weight, 4810.52 Da) is a 39 amino acid linear peptide conjugated to a C20 fatty diacid moiety [diacid-γ-Glu-(AEEA)2] via a linker connected to the lysine residue at position 20. The peptide sequence contains two non-coded amino acid residues at positions 2 and 13 (α-amino isobutyric acid, Aib), and the C-terminus is amidated [238]. The Aib residues protect the surrounding peptide bonds against proteolysis. The blue circle represents the amino acid homologous with GIP; the orange circle represents the amino acid unique to tirzepatide; the green circle represents the amino acids homologous with both GIP and GLP-1, and the violet circle represents the amino acid homologous with GLP-1 and exendin. (**B**) Native GLP-1 and GIP amino acid sequences and schematic view of relative receptors. GLP-1 and GIP are two gut hormones (incretins) secreted from L and K enterocytes, respectively, in response to digested nutrients. Endogenous GLP-1 is mainly produced as GLP-1(7–36NH2), with a low proportion produced as GLP-1(7−37) and GLP-1(1−37) or GLP-1 (1–36NH2) [239]. GLP-1 has a half-life of ~ 2–3 min and is subjected to rapid in vivo proteolysis by the dipeptidyl peptidase-IV (DPP-IV) and fast renal elimination. Most circulating GIP is GIP(1−42), cleaved from pro-GIP. GIP is quickly cleared from circulation with a half-life of 4 min [240]. GLP-1 binds the GLP-1 receptor (GLP-1R), a class B family's seven transmembrane G protein-coupled receptor (GPCR). GLP-1R acts primarily via the Gαs pathway (with increased cAMP production) and can also stimulate the Gαq and β-arrestin pathways. GIP promotes its biological action by binding with the GIP receptor (GIPR), a class of B GPCR, acting similarly to GLP-1R. (**C**) Overview of GLP-1 and GIP direct biological effects. GLP1 and GIP potentiate meal insulin secretion from the pancreas and regulate glucagon release. The incretin receptors are widely distributed in other organs and tissues beyond the pancreas, where they exert pleiotropic activities. GLP-1 reduces appetite by acting on the hypothalamus and the nucleus of the solitary tract and altering neural activity in other areas of the central nervous system, including the reward system. Conversely, the anorexigenic effect of GIP is controversial and not fully elucidated. It has been proposed that GIP enhances the satiating effect of GLP-1 by expanding its tolerance window through an anti-emetic effect in the brainstem [241]. In addition, GLP-1 owns cardioprotective properties, ameliorating cardiac metabolism (*e.g.*, increased glucose utilization and decreased lipid metabolism) and inflammation. The role of GIP in cardiovascular system regulation is now under investigation; the GIPRs are expressed on endothelial cells, and their activation attenuates aortic inflammation and atherosclerosis. Preclinical studies show that GIPR reduces cardiac hypertrophy and fibrosis in mice with experimental hypertension; in contrast, cardiomyocyte GIPR deletion is cardioprotective in mice with ischaemic cardiac injury secondary to coronary artery occlusion [242]. GLP-1 and GIP receptors are found on human EAT with possible beneficial cardiovascular effects (i.e., reduced inflammation, increased FFA oxidation, induction of browning). Ongoing clinical trials are evaluating the cardiovascular safety of tirzepatide (see text). GLP-1 and GIP have opposite effects on lipid metabolism in WAT. While GLP-1 stimulates lipolysis and inhibits lipogenesis, GIP promotes adipocyte lipid storage and increases blood flow to adipose tissue, expanding the white fat. GIP also affects the secretion and circulating levels of adipokines and proinflammatory cytokines. GIPR antagonists have been developed based on these results, showing only little anti-obesity properties compared to GIPR agonists. How pharmacological activation and inhibition of GIPR improve energy and lipid metabolism is under investigation. It has been hypothesized that GIPR agonists may desensitize GIP receptor signaling or that GIPR agonists and antagonists improve metabolism through independent mechanisms [239]. GIP and GLP-1 can also ameliorate liver (i.e., decreased glucose production and steatosis) and muscle (i.e., increased insulin sensitivity and glucose uptake) metabolism. However, these beneficial effects are not due to direct effects on the organs, since the liver and muscle do not express GLP-1 and GIP receptors, but are linked to indirect signalings, such as changes in circulating concentrations of insulin and glucagon, and the influence on metabolic substrate supply to liver and muscle. (**D**) Tirzepatide modulates the amino acid profile in plasma and BAT in a direction consistent with improved metabolic health. Chronic tirzepatide treatment significantly decreased the BCAA plasma levels and the metabolites generated by their catabolism (BCKAs, including 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, and alpha ketoisovaleric acid, as well as 3-hydroxyisobutyrate, and 2-hydroxyisocaproic acid) in T2D subjects. Decreased BCAA levels were accompanied by significant increases in circulating glycine, serine, threonine, glutamine, and asparagine [229]. A recent study in diet-induced obese mice demonstrated that chronic 14 day-treatment with tirzepatide specifically impacts amino acid levels in BAT. The BAT amino acid signature was reminiscent of the amino acid profile in BAT following cold exposure or EAA-based diet consumption when amino acid uptake is increased to achieve optimal thermogenic capacity. Furthermore, tirzepatide stimulates BCAA catabolism and mitochondrial activity, with increased TCA cycle intermediates, in brown fat [231]. Created using BioRender.com.

energy [258]. Moreover, administering GCG to animals without functional BAT their oxygen consumption increases. This result suggests that mechanisms independent of BAT also contribute to GCG-mediated EE. Recent studies indicate that GCG may indirectly mediate its metabolic effects, including EE, through FGF21. FGF21 is a protein belonging to the FGF superfamily and is synthesized mainly by the liver and other tissues. It has broad metabolic effects, such as promoting EE, weight loss, improved lipid metabolism and glycemia [261]. Some studies have found FGF21 to play a role in increased sympathetic outflow to BAT and WAT tissue. This leads to a boost in lipolysis and expression of thermogenetic markers like UCP1 [262–264]. Other findings indicate that there are mechanisms in EE driven by central FGF21 signaling that are not dependent on UCP1. Specifically, mice that lacked the central β-klotho receptor (KLB), the necessary co-receptor for FGF21, experienced only a partial reduction in weight loss when treated with chronic GCG (IUB288, acyl-glucagon). Surprisingly, it was observed that KLB antagonism did not impact the expression of BAT UCP1 in response to GCG therapy. Overall, the studies mentioned above suggest that GCG signaling may activate a combination of energy-consuming pathways, improving the metabolic rate. Hope and Tan [251] recently compiled a summary on the impact of GCG on EE in humans, focusing on Nair and colleagues' initial study of insulin-treated individuals with T1DM. That study revealed a significant rise in metabolic rate upon discontinuing insulin therapy, which was attributed to the correlation between plasma GCG levels and metabolic rate. It was suggested that the increase in EE could be linked to the counter-regulatory hormone GCG, which stimulates protein turnover and gluconeogenesis [265]. Multiple studies have shown that GCG may increase EE in humans, indicating that protein breakdown plays a role in the GCG-stimulated resting metabolic rate [266–270]. This was shown in studies where GCG was infused along with leucine, which was labelled with stable isotopes, while the subjects underwent indirect calorimetry. The results showed that GCG infusions during somatostatin co-infusion led to increased leucine oxidation and resting metabolic rate, and these outcomes were positively correlated with plasma GCG levels [271].

The established ability of GCG to increase hepatic amino acid catabolism has been reported in various studies. In rodents, GCG has been found to enhance hepatic urea cycle activity and increase urinary nitrogen excretion [272–274], by increasing the expression of hepatic amino acid transporters and N-acetyl glutamate synthetase [275]. Unlike lean subjects, obese individuals with hepatic steatosis fail to respond with the suppression of circulating amino acids to GCG infusions [276]. The significant connection among glucagon, amino acids, and lean body mass can be observed through the glucagonoma syndrome [277,278]. Muscle tissue is not believed to possess the GCGR [279], and it has been suggested that GCG-induced loss of lean mass occurs indirectly through hepatic catabolism induced by GCGR and subsequent reductions in plasma amino acid levels, resulting in altered intramuscular amino acid sensing through mTORC1 and ATF4 [278,280–283]. Nonetheless, the mechanisms that control EE following the development of hypoaminoacidemia stimulated by GCG are poorly understood. Hope and Tan [251] recently presented an amino acid-focused model contributing to the GCG-modulated EE. Activation of the hepatic GCGR, either through prolonged GCG infusion or long-acting GCGR agonists, has been found to increase hepatic amino acid uptake and catabolism. This can cause hypoaminoacidemia, which may trigger an increase in hepatic FGF21 production and release, signaling the central nervous system to raise EE. Hypoaminoacidemia may alter whole-body protein turnover and amino acid metabolism through amino acid sensing pathways, increasing energy consumption. This process may also impact amino acid metabolism

in thermogenic adipose tissues, with potential implications for metabolic multiagonists targeting the GLP1R-GIPR-GCGR pathways.

Even though GCG infusion and cold exposure increased EE to a similar extent, only cold exposure led to increased BAT ^{18}F-FDG uptake and supraclavicular temperature in humans when studied using PET technology [245]. In contrast, research on unimolecular triple agonists for GLP1R/GCGR/GIPR, such as SAR441255 [284] and Retatrutide (LY3437943) [285–288], have demonstrated better weight loss results than dual agonists [289], thanks to an increase in EE that is not fully understood. Interestingly, the maximally effective triple agonist and tirzepatide (and other co-agonist) groups showed similar reductions in food intake (although only the former led to a markedly elevated EE) [289].

10. The case of epicardial adipose tissue

We have recently reported the presence of GLP-1R, GIPR, and GCGR in human epicardial adipose tissue (EAT) [290,291]. EAT is a specialized type of fatty tissue found between the heart muscle and the visceral layer of the outer membrane of the heart [292]. EAT is a unique fat depot due to its unobstructed proximity to the underlying myocardium and peculiar transcriptome [293]. Primarily made of white adipocytes, it may also include features resembling brown or beige fat [294]. Under normal circumstances, EAT plays a protective role. EAT supplies free fatty acids (FFAs) to the nearby myocardium and acts as a buffer, safeguarding the heart against elevated FA levels [295]. EAT is believed to be a direct source of heat for the myocardium and protects the heart during unfavorable haemodynamic conditions such as ischaemia or hypoxia [296]. The control processes for thermogenesis in EAT are complex and require further understanding [297]. As individuals age, EAT brown fat-like activity decreases significantly, and the presence of white adipocytes increases [294]. This shift from brown to beige fat is observed in adults. However, long-term ischemic conditions, such as advanced stages of coronary artery disease (CAD), can also decrease brown fat-like activity in EAT [297]. In patients with advanced CAD, a decrease in the expression of genes that encode proteins involved in adipocyte browning and thermogenic activation occurs in EAT; in contrast, the expression of genes that encode pro-inflammatory cytokines is increased [298]. Advanced CAD can also be associated with apoptotic and fibrotic changes within epicardial adipocytes [299]. These structural and functional changes in the human EAT can be clinically detected with traditional and novel imaging techniques [292]. It would be desirable to induce EAT to resume its function like brown fat, which can offer significant heart benefits. Research has shown that increasing the expression of genes involved in BAT activation and mitochondrial signaling through pharmacological approaches in EAT can reduce left ventricular mass and inflammation [300].

EAT secretome and proteome include molecules regulating inflammation, fibrosis, coagulation, immune response, and thermogenesis. Although a specific role in amino acid metabolism has not been explored yet, EAT expresses factors directly involved in cellular function. As compared to subcutaneous WAT, EAT is highly enriched in genes encoding for cell-cell signaling, cell-cell adhesion, apoptosis, intramolecular oxidoreductase activity, and RNA polymerase II transcription factor function [298]. Genes encoding for acid-amino acid ligase and acid-amino acid ligase activity are down-regulated in EAT from patients with CAD [298]. Ligase catalyzes the ligation of an acid to an amino acid via a carbon-nitrogen bond, with the concomitant hydrolysis of the diphosphate bond in ATP. We speculate that the downregulation of ligase activity in pathological EAT can be associated with a lower ATP production in EAT, a brown-like adipose physiologically producing very low ATP quantities. Interestingly both EAT mitochondrial function and BAT-like thermogenic activity decrease with advanced CAD and aging, and low mitochondrial respiration in EAT was associated with CAD severity [301]. Pharmacological targeting of EAT genes involved in amino acid metabolism may influence adipocyte mitochondrial activity and heat production. Further studies will be necessary to check this hypothesis.

Of note, leveraging a unique large clinical trial of noninvasive imaging in patients with stable chest pain (i.e., the PROMISE study), it has been found that total circulating BCAAs are associated with body mass index (BMI), hepatic steatosis (HS), and EAT volume. However, association with HS remained significant after adjustment of covariables, notably BMI, BCAAs were not in the causal pathway of either HS or CAD [302]. BCAA associations with EAT were likely primarily related to total body adiposity (and not EAT specifically), given attenuation in models inclusive of BMI.

Given its measurability and responsiveness to pharmacological agents modulating the fat, EAT is considered a new therapeutic target [303]. Notably, we identified the GIPR and GCGR with an immunostaining analysis of EAT samples from patients undergoing elective coronary artery bypass graft (CABG) surgery for CAD and those undergoing valve replacement (VR) surgery. In particular, we observed GIP and GCG receptors in the isolated and crown-like structures (CLS) macrophages, with an intense positivity in the wall, including endothelial cells and pericytes, of capillaries and pre-capillary vascular structures. On the contrary, only a variable cytoplasmic immunoreactivity for either receptor was evident in differently-sized mature adipocytes. CLS density is considered a marker of adipose inflammation [304]. Of note, multiple recent studies implicate macrophages in regulating thermogenic, sympathetic neuron-mediated NE signaling in adipose tissues [305]. The presence of the receptors and the vicinity to the myocardium suggest the EAT can receive and mediate the beneficial cardiovascular effects of these drugs. Interestingly, GLP1R agonists can reduce EAT inflammation and increase FFA oxidation as fuel for the myocardium, and induce fat browning, and GLP1R-dependent browning effect on EAT can lead to improved myocardial insulin sensitivity and metabolism [306].

Another important class of new cardio-metabolic drugs, sodium-glucose transporter 2 (SGLT2) inhibitors, can induce EAT ketogenesis and reduce oxygen consumption in failing hearts [307].

EAT is a unique fat depot that regulates myocardial metabolism and function. Pharmacological manipulation of EAT can restore its physiological properties and improve heart performance.

11. Adipose tissue macrophages, amino acids, and thermogenesis

Macrophages are a group of immune cells with varying functions in responding to immune and homeostatic situations. They can change their functional phenotype through a polarization process, enabling them to respond to local microenvironmental stimuli. Different markers expressed on their surface, the secretion of specific cytokines, and metabolic adaptations define macrophages and their functional phenotype classification. Generally, macrophages are grouped into two categories based on the gene expression for protein markers: classically activated, pro-inflammatory or M1 macrophages [308,309], and alternatively activated, anti-inflammatory, or M2 macrophages [310,311] (Fig. 6A).

The presence of amino acids is a critical component for a successful immune response, and a lack of amino acids during an immune reaction or inflammation can lead to weakened immune cells that are unable to complete their functions, including cell division, maturation, migration, and the completion of effector functions. Consequently, macrophage adaptation to rapidly changing nutrient sources often requires the selective utilization of amino acid catabolism to sustain their immune activity and overall activation [314] (Fig. 6A). The availability of amino acids plays a key role in controlling various macrophage response pathways, such as mTOR signaling and NO production. When exposed to proinflammatory stimuli, such as LPS, TNF-α, or IFN-γ, overexpression of iNOS (NOS2) occurs, leading to the redirection of arginine towards the production of NO and citrulline [318]. NO inhibits the repolarization

between M1 and M2, while citrulline is utilized by argininosuccinate synthase 1 to generate argininosuccinate, which is immediately metabolized to recover arginine and maintain the production of NO [319]. The anti-inflammatory M2 macrophages produce ornithine and urea through the catabolism of arginine by over-expressing arginase 1 (ARG1), in contrast to M1 macrophages. Ornithine is converted by ornithine decarboxylase into a group of compounds called polyamines (putrescine, spermidine, and spermine). These polyamines are crucial for regulating cell growth and play an important role in tissue repair. Intriguingly, the activation of ARG1 in macrophages induces an anti-inflammatory phenotype, leading to a decrease in T-cell proliferation and cytokine production [320,321]. In addition, tryptophan metabolism is essential for maintaining peripheral immune tolerance. Macrophages rely heavily on extracellular sources of glutamine and consume significant amounts of this amino acid (for references, [314]) (Fig. 6A).

Exposure to cold temperature rapidly promotes adipose tissue M2 macrophage (ATM) proliferation, which secrete catecholamines to induce thermogenic gene expression in BAT [315] (Fig. 6B). Impaired metabolic adaptations to cold were observed due to the lack of M2 macrophages. On the other hand, interleukin-4 (IL4) administration increased thermogenic gene expression, fatty acid mobilization, and EE; these changes were macrophage-dependent. While the results of Fischer et al. [322] may not have provided adequate support for the involvement of alternatively activated macrophages in catecholamine synthesis or their contribution to adipose tissue adaptive thermogenesis, various discoveries still reinforce this hypothesis. Mutagenesis experiments of BAT $Cx3Cr1^{+}$ macrophages impaired thermogenesis homeostasis without affecting the ability to generate heat in response to cold. In pre-obese mice with mutant macrophages, a malfunction of BAT was found to be linked to reduced amounts of sympathetic innervation and local concentration of NE. Because of this, adipocytes had lower levels of thermogenic factors [323]. SAMs, or sympathetic neuron-associated macrophages, were found to be a group of cells that facilitate the removal of NE by producing two proteins - an NE transporter called solute carrier family 6 member 2 (Slc6a2) and a degradation enzyme called monoamine oxidase A (MAOA) [324] (Fig. 6C). Activation of the SNS through optogenetics increased the uptake of NE by SAMs and resulted in a shift of the SAM profile toward a proinflammatory state. Two mouse models of obesity also exhibited a greater proportion of SAMs in the SNS. Additionally, adipose deletion of cannabinoid receptor type-1 (CB1) decreased body weight, reduced total adiposity and insulin resistance, and enhanced BAT thermogenesis beyond the browning of white adipocytes significantly; these modifications were linked with an upsurge in M2 macrophages and an improved sympathetic tone in adipose tissue [325]. M2-like macrophages in adipose tissue have been found to release the cytokine Slit3, which promotes thermogenesis and sympathetic innervation in mice [326]. Slit3 binds to the ROBO1 receptor, activating Ca^{2+}/calmodulin-dependent protein kinase II signaling and NE release from sympathetic terminals, improving adipocyte thermogenesis. Transplanting M2 macrophages that overexpress Slit3 to subcutaneous WAT enables beiging and thermogenesis, while mice deprived of Slit3 in their myeloid cells have a lower tolerance for cold and gain weight more quickly. Male mice without the AIF1 protein were found to be resistant to obesity and hyperglycemia caused by HFD in recent research [327]. This phenotype was related to elevated levels of NE in adipose tissue, which resulted from reduced expression of MAOA and decreased NE clearance by macrophages that lack AIF1. Interestingly, variants in human sequences around the AIF1 gene locus are related to obesity and diabetes. This identifies AIF1 as a controller of MAOA expression in macrophages and catecholamine activity in adipose tissues, which leads to reduced EE and increased energy storage, potentially contributing to obesity in humans.

Only a limited number of research studies have focused on studying the impacts of amino acids on ATMs. Recently, Wang et al. [71] found that leucine deprivation reduced ATM accumulation – with decreased $CD11c^{+}$ (M1) and increased $CD11c^{-}$ (M2) in ATMs –, increased browning, and stimulated lipolysis in WAT. Mechanistically, leucine deprivation activated GCN2 signals in macrophages. GCN2 is a critical regulator of cellular responses under amino acid deprivation [328]. Myeloid-specific deletion of GCN2 in mice blocked leucine deprivation-induced browning and lipolysis in WAT. Further analyses revealed that GCN2 activation in ATMs reduced the expression of MAOA, resulting in increased NE secretion from macrophages to adipocytes.

Overall, these results suggest that the GCG receptor-targeted multiagonists might activate sympathetic innervation and thermogenic activity of BAT and beige fat controlling the amino acid metabolism and, consequently, the NE-releasing or NE-clearing ATM accumulation and function in these fat depots.

12. Summary and future directions

The studies summarised here provide new insights into adaptive thermogenesis and its drug and nutritional modulation as therapy for obesity and related disorders. Diverse subpopulations of brown and beige adipocytes with distinct molecular and metabolic features within single adipose depots have been described. Given these distinct molecular profiles of preadipocytes and differentiated adipocytes, it is likely that the cellular lineages of these subpopulations are determined at the precursor stage. Still, future studies are needed to delineate the adipocyte precursor heterogeneity. Adipose thermogenesis can be enhanced through increased activity of existing thermogenic fat and recruitment of more functional thermogenic fats; the two mechanisms may inform differently amino acids and/or GLP1, GIP, and GCG actions in different cell types.

Mounting evidence supports the critical relevance of amino acid metabolism in physiology and pathophysiology of thermogenic fat. Most importantly, metabolic fluxes and liquid chromatography-tandem mass spectrometry analysis demonstrate that circulating and adipose tissue amino acid profiles are much more than correlative aspects of BAT/beige biology; the unique combinations of EAAs and NEAAs may be causal in modulating cell biochemistry, organelle architecture, epigenetic control of cell development, energy metabolism, and secretory function. Bioinformatics, artificial intelligence, and machine-learning tools will dissect these topics appropriately. In these approaches, computer systems use large amounts of data (from Biobanks) to build predictive statistical models that are iteratively improved by incorporating new data. Deep learning, a powerful subset of machine learning that includes the use of deep neural networks, has had high-profile applications in image object recognition, in addition to voice recognition, autonomous driving, and virtual assistance [329]. These approaches are now being applied to yield clinically directive medical information. We imagine that these tools will allow us to go beyond the present elaboration limits of iterative prediction of disease risk through computer simulation of plasma and tissue aminograms [330,331], and lead to proposals of amino acid combinations in dietary supplements ameliorating metabolic diseases.

Novel mass spec-based tracing of metabolite fates at subcellular resolution and in a spatiotemporal manner, as well as new proteomics, single-cell and single-nucleus omics, RIBOmap, and induced pluripotent stem cell-based organoids, are revealing spatial patterns in translation at a very high resolution to look at individual cells within adipose tissues and discover how different adipose cell types are regulating translation differently. Scientific developments in these roads will facilitate understanding the mechanisms underlying amino acid regulation of thermogenic fat at a better resolution.

Moreover, the findings, summarised here, highlight the potential involvement of amino acid profiles in ATM-induced thermogenic adipocyte activation by GLP1-GIP-GCG receptor multiagonists in subjects with metabolic disorders. Key questions remain, including: 1) the mechanistic details of GLP-1-, GIP-, and GCG-regulated amino acid

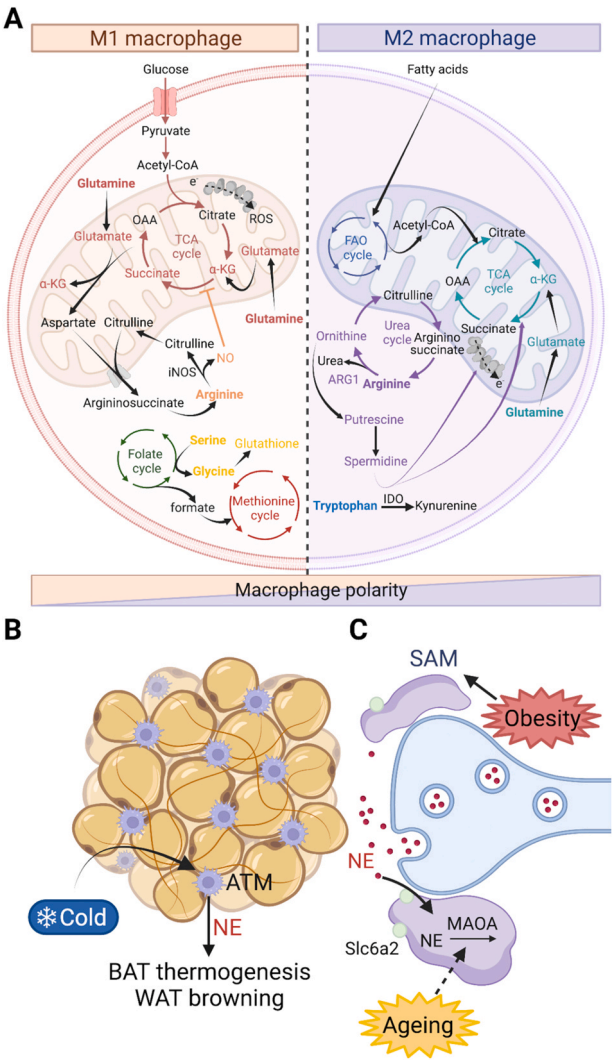

Fig. 6. Amino acids regulate macrophage polarity and represent a valuable tool in the stimulation of the thermogenesis program via the M2 activation. (**A**) The M1/M2 model classifies the macrophage polarization state: the classically activated macrophages (M1), induced by pro-inflammatory stimuli (*e.g.*, LPS or IFNγ), which specifically express inducible nitric oxide synthase (iNOS) enzyme; the alternatively activated macrophages (M2), induced by anti-inflammatory stimuli (*e.g.*, IL-4 or IL-13), which specifically express arginase 1 (ARG1). Amino acids and their metabolism are involved in the functional regulation of the macrophage. Besides the iNOS/ARG1 axis, the mitochondrial TCA cycle represents a central immune-metabolic hub for macrophage activation. Under pro-inflammatory stimuli, M1 macrophages show upregulation of glycolysis and decreased mitochondrial oxidative phosphorylation (i.e., TCA cycle activity), with ETC rapid remodeling, which causes an increased ROS production and drives the inflammatory response. The M1 macrophages express iNOS that converts arginine into citrulline and NO, favoring a robust inflammatory response. Acute exposure to NO inhibits the ETC and TCA cycle with intermediates' accumulation (citrate, succinate, and itaconate) in the early stage of polarization; it promotes the loss of mitochondrial complexes during the late stages of polarization. Recently, it has been proposed that the M1 macrophages fail to repolarize to an M2-like phenotype because M2 polarization is highly dependent on a functional ETC [312]. Moreover, a high amount of NO leads to decreased carbon flux from citrate to α-ketoglutarate (α-KG) and triggers increased carbon entry from glutamine into the TCA cycle to fuel α-KG and succinate anaplerosis. The α-KG/succinate ratio seems to be a key determinant of the inflammatory response. Serine is also involved in M1 polarization, increasing glycine production and folate cycle with the increase in glutathione production and methionine cycle, which sustain the inflammatory response. In particular, glutathione restores IL1β during inflammation [313]. Compared to M1, M2 macrophages show a functional TCA cycle and enhanced oxidative phosphorylation (OXPHOS), mainly using fatty acids and glutamine as substrates instead of glucose. Furthermore, M2 macrophages express ARG1 that converts arginine to ornithine, providing the substrates for polyamine synthesis (i.e., putrescine and spermidine), involved in mitochondrial activation and M2 function. Another critical amino acid for M2 polarization is glutamine. Besides its role in the TCA cycle and OXPHOS support, it is also critically involved in the M2 activation [313]. Tryptophan metabolism regulates peripheral immune tolerance; its metabolites, such as kynurenine, suppress T cell immunity. In macrophages, the indoleamine 2,3-dioxygenase (IDO) represents the limiting enzyme of tryptophan catabolism. Though pro-inflammatory stimuli (e.g., IFN-γ, TNF-α, prostaglandins) induce IDO expression, macrophages show an M2 phenotype [314]. (**B**) The adipose tissue M2 macrophages (ATMs) coordinate the adaptive thermogenic program by increasing BAT thermogenic capacity and WAT browning and mobilizing fatty acids to fuel uncoupled respiration, increasing EE. Thermogenic stimuli promote the NE release from ATMs, and the ATM-released NE allows for coordinating the adaptive thermogenesis in response to diverse stimuli as a second parallel circuit in addition to the SNS recruitment [315]. (**C**) Recent studies have identified a unique subpopulation of pro-inflammatory macrophages in adipose tissue (sympathetic neuron-associated macrophages or SAMs) regulating the sympathetic tone of fat tissues. The SAMs are localized around sympathetic neurons, express the Slc6a2 transporter for NE uptake and monoamine oxidase A (MAOA) for NE degradations, and remove NE from the interstitial space. Obesity increases the SAM abundance in adipose tissue, decreasing NE signaling and impairing BAT thermogenesis and WAT browning during cold exposure [316]. Accordingly, the genetic ablation of Slc6a2 in SAMs improves BAT function and WAT browning, leading to weight loss in obese mice [316]. During ageing, the SAMs show highly activated MAOA that dampens SNS activity and lipolysis in adipose tissue, contributing to reduce fatty acid availability to counteract starvation and exercise tolerance [317]. Created using BioRender.com.

metabolism; 2) the structure-efficacy of GLP-1R, GIPR, and GCGR agonists and activation of immune adipose response; 3) the mechanistic factors modulating the brain-dependent adipose control by the new anti-obesity drugs; and 4) the influence of these drugs (and innovative dietary approaches) on intestinal microbiota composition and microbiota metabolites regulating BAT/beige thermogenesis and metabolic homeostasis. Testing the extent to which these are general principles in human adipose tissue function and systemic metabolic homeostasis will be an interesting objective for the field.

CRediT authorship contribution statement

Chiara Ruocco, Alessandra Valerio, and Enzo Nisoli: Conceptualization, Writing – original draft; **Chiara Ruocco, Alexis Elias Malavazos, Maurizio Ragni, Michele O. Carruba, Alessandra Valerio, Gianluca Iacobellis, and Enzo Nisoli:** Writing – review & editing. All the authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

None.

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