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Treat more than heat—New therapeutic implications of *Cimicifuga racemosa* through AMPK-dependent metabolic effects

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ABSTRACT

Background: *Cimicifuga racemosa* extracts (CRE) have obtained a "well-established use status" in the treatment of postmenopausal (*i.e.*, climacteric) complaints, which predominantly include vasomotor symptoms such as hot flushes and sweating, as well as nervousness, irritability, and metabolic changes. Although characteristic postmenopausal complaints are known for a very long time and the beneficial effects of CRE on climacteric symptoms are well accepted, both the pathophysiology of postmenopausal symptoms and the mechanism of action of CREs are not yet fully understood. In particular, current hypotheses suggest that changes in the α-adrenergic and serotonergic signaling pathways secondary to estrogen depletion are responsible for the development of hot flushes.

Purpose: Some of the symptoms associated with menopause cannot be explained by these hypotheses. Therefore, we attempted to extend our classic understanding of menopause by integrating of partly age-related metabolic impairments.

Methods: A comprehensive literature survey was performed using the PubMed database for articles published through September 2021. The following search terms were used: (cimicifuga OR AMPK) AND (hot flush* OR hot flash* OR menopaus* OR osteoporos* OR cancer OR antioxida* OR cardiovasc*). No limits were set with respect to language, and the references cited in the articles retrieved were used to identify additional publications.

Results: We found that menopause is a manifestation of the general aging process, with specific metabolic changes that aggravate menopausal symptoms, which are accelerated by estrogen depletion and associated neurotransmitter dysregulation. Cimicifuga extracts with their metabolic effects mitigate climacteric symptoms but may also modulate the aging process itself. Central to these effects are effects of CRE on the metabolic key regulator, the AMP-activated protein kinase (AMPK).

Conclusions: As an extension of this effect dimension, other off-label indications may appear attractive in the sense of repurposing of this herbal treatment.

Abbreviations: AICAR, 5-aminoimidazole-4-carbox-amide-1-β-d-ribofuranoside; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; AMPKx, isoform x of AMPK; ARC, arcuate nucleus of the hypothalamus; AUC, area under the curve; ATP, adenosine triphosphate; BAT, brown adipose tissue; BDNF, brain-derived neurotrophic factor; BMD, bone mineral density; CaMKK, calmodulin-dependent kinase I kinase; CHD, coronary heart disease; 95% CI, 95% confidence interval; CNS, central nervous system; CRE, *Cimicifuga racemosa* extract; DNA, deoxyribonucleic acid; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERRα, estrogen-related receptor alpha; fMRI, functional magnetic resonance imaging; HIF-1α, hypoxia-inducible factor 1α; HMPC, Herbal Medicinal Products Committee of the European Medicines Agency; HF, hot flushes; HOMA-IR, homeostasis model assessment of insulin resistance; HRT, hormone replacement therapy; H_x, histamine receptor x; 5-HT_x, 5-hydroxytryptamine receptor x; IL-6, interleukin 6; KMI, Kupperman Menopausal Index; LH, luteinizing hormone; LHA, lateral area of the hypothalamus; LKB1, tumor suppressor liver kinase B1; MRS, Menopause Rating Scale; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; OGTT, oral glucose tolerance test; OVX, ovariectomized; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; PI3, phosphoinositide 3-kinase; PKA, protein kinase A; *p.o.*, peroral administration; POA, preoptic area of the hypothalamus; PVH, paraventricular nucleus of hypothalamus; RANKL, receptor activator of NF-κB ligand; ROS, reactive oxygen species; RR, relative risk; RCT, randomized controlled trial; SERM, selective estrogen receptor modulator; SIRT1, sirtuin-1; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; SNS, sympathetic nervous system; SWAN, Study of Women's Health Across the Nation; TAK1, TGFβ-activated kinase; Tc, core body temperature; TNFα, tumor necrosis factor α; UCP1, uncoupling protein 1; VEGFR-1, vascular endothelial growth factor receptor-1; VMH, ventromedial nucleus of the hypothalamus; WHI, Women's Health Initiative study.

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Introduction

Menopause marks the transition from the reproductive phase to the non-reproductive phase in women. The primary symptoms include hot flushes (HF; also known hot flashes), with associated symptoms such as reddening of the skin, a sudden sensation of heat or warmth, and (night) sweating, as well as insomnia, nervousness, irritability, and palpitations. In addition, postmenopausal women often experience a change in libido, dyspareunia (painful intercourse), depression, musculoskeletal pain, vaginal pruritus and dryness, and various changes in metabolism (Table 1). These metabolic changes are also typically associated with an increase in body mass gain, an increased risk of diabetes, pain and/or inflammation, and increased bone turnover with an enhanced risk of developing osteoporosis (Burger et al., 2002; Crandall et al., 2011; NAMS, 2004).

In addition to causing discomfort and a decreased quality of life, menopause causes considerable costs associated with medication, medical procedures, and sick leave from work. For example, a database including more than 750,000 female employees in the US showed that menopausal women incurred 40% higher health-related costs compared to non-menopausal age- and employment-matched controls (Kleinman et al., 2013).

Currently, the typical menopause-related complaints and increased risk of the above-mentioned diseases are generally attributed to the significantly reduced estrogen levels that characterize the transition during menopause at the hormonal level (Kaunitz and Manson, 2015; Thacker, 1997). This notion has been largely confirmed in clinical settings of estrogen withdrawal, for example during treatment of breast cancer with the estrogen receptor antagonist tamoxifen, which induces a state of "pharmacological menopause" with symptoms similar to climacteric (*i.e.*, menopausal) symptoms (Benshushan and Brzezinski, 1999). Furthermore, hormone replacement therapy (HRT) is considered the most effective strategy to reduce major menopausal complaints, albeit with an increased risk of developing cardiovascular diseases, diabetes, breast cancer, and/or endometrial cancer (Anagnostis et al., 2019; Newson, 2018; Paciuc, 2020; Sjögren et al., 2016; Stuenkel, 2017). However, this view on menopause is limited to hormone levels and does not usually include the effects of aging, which also increase the risk of many of the conditions and diseases attributed to menopause, including weight gain and higher risk of diabetes, cardiovascular diseases, inflammatory disease, osteoporosis, and others. In addition, established treatment options are available that reduce menopausal complaints and associated disorders, independent of estrogen levels. For example, *Cimicifuga racemosa* has been shown to have beneficial effects on hot flushes and metabolic dysregulation, independent of estrogen receptor signaling (Drewe et al., 2015; Rabenau et al., 2021; Seidlova-Wuttke, 2010), thus calling to question our current understanding of the mechanisms that underlie menopausal complaints and extending potential treatment options beyond the perspective of hormone levels.

Cimicifuga racemosa extracts (CRE) have traditionally been used as a folk remedy to treat climacteric symptoms (Wichtl et al., 2004). Moreover, several randomized controlled trials (RCTs) have confirmed the clinical efficacy of CRE in postmenopausal women with climacteric symptoms (Bai et al., 2007; Liske et al., 2002; Osmers et al., 2005; Schellenberg et al., 2012; Stoll, 1987; Wuttke et al., 2003b). These effects have been described in detail in several monographs as pharmacologically active treatment (ESCOP, 2003; WHO, 1996), and several systematic reviews and meta-analyses have been published (Drewe et al., 2015; Henneicke-von Zepelin, 2017; Sarri et al., 2017; Shams et al., 2010). In 2010, the community herbal monograph of the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA, 2017, 2018) granted "well-established use" status to CRE.

In this review, we summarize the emerging evidence that has extended the current view on regarding the mechanisms that underlie menopause and menopause-related disorders to include general systemic age-related changes, and, in particular changes in cellular metabolism. We suggest that the causal factors underlying menopausal symptoms may be better explained by these age-related metabolic changes, including accelerated oxidative dysregulation. This extended view can help improve our understanding of the mechanism of action of CREs in relieving climacteric symptoms. Last but not least, this mechanism of action may create new therapeutic options for other indications by repurposing *Cimicifuga* preparations.

Table 1
Summary of common menopausal symptoms.

Vasomotor symptoms	Vaginal symptoms	CNS symptoms	Other symptoms
Hot flushes	Atrophy	Sleep disturbances	Weight gain
Sweating	Dryness	Nervousness/ paresthesia	Bloating
Palpitations	Itching	Depressive symptoms	Joint pain/ swelling
Vertigo	Dyspareunia	Cognition impairment	
		Decreased libido	
		Anxiety	

Collected from: (Freeman et al., 2007; Heinemann et al., 2003; Kupperman et al., 1959) , **bold** = core symptoms.

Methods

Literature search methodology

We searched the PubMed database for articles published through September 2021 using the following search terms: (cimicifuga OR AMPK) AND (hot flush* OR hot flash* OR menopaus* OR osteoporos* OR cancer OR antioxida* OR cardiovasc*), with no limits set for language. Our initial search yielded 7898 hits, and references cited in the papers retrieved were used to identify additional articles.

Results

The results are structured as follows: In the first subsection, the current state of knowledge on the physiology of temperature regulation and the pathophysiology of hot flushes is described and the tissue-specific pathophysiologic involvement of AMPK in energy metabolism and in the formation of major menopausal symptoms is discussed. In the second subsection, defined and clinically used *Cimicifuga racemosa* extracts are described including their main constituents, and their preclinical and clinical pharmacokinetics and pharmacology. The third subsection develops the extended view of the mechanism of action and how this gives rise to new potential clinical applications for *Cimicifuga racemosa* extracts.

Temperature regulation and hot flushes

Body temperature is sensed by afferent heat-sensitive fibers in the gastrointestinal tract, intra-abdominal veins, spinal cord, and skin, which project their information to the hypothalamus. In response to peripheral input, heat-sensitive neurons control the release of heat *via* peripheral vasodilation and sweating. The functionally coupled elements of these thermoregulatory control loops are under catecholaminergic and serotonergic control (Rossmanith and Rubberdt, 2009). Core body temperature (Tc) is regulated between an upper threshold by sweating and a lower threshold by shivering. Within this range known as the thermoneutral trigger zone in which heat production by metabolism is sufficient to maintain Tc (González-García et al., 2017), no adjustment of the Tc regulatory system is necessary (Archer et al., 2011). When the ambient temperature decreases, heat-saving strategies such as peripheral vasoconstriction, piloerection, and shivering are triggered, followed by facultative or adaptive thermogenesis *via* active heat production in

brown adipose tissue (BAT) (Contreras et al., 2015, 2016; González-García et al., 2017; López, 2018b; López et al., 2015; Martínez de Morentin et al., 2014; van Dam et al., 2015). In contrast, when the ambient temperature increases, heat-dissipating mechanisms such as peripheral vasodilatation and sweating are triggered.

In symptomatic perimenopausal women, the thermoneutral trigger range becomes considerably more narrow; thus, only a small elevation in Tc can trigger the initiation of hot flushes in these women, but not in non-menopausal controls (Freedman, 2014; Freedman and Krell, 1999). This change in the trigger range can be regarded as an increased sensitivity of the heat-dissipation response to a small change in temperature (Rance et al., 2013). Interestingly, hot flushes are associated with a marked increase in metabolic rate reflected by an increase in the respiratory exchange ratio that increases temperature itself (Freedman, 1998), as well as peripheral vasodilation and sweating serving to dissipate heat (Freedman, 2014). Brain imaging studies using functional magnetic resonance imaging (fMRI) during the onset of a hot flush have shown activation in the insular and prefrontal cortex following "pre-hot flush" activation in the brainstem, possibly reflecting the functional origin of thermogenic activity (Archer et al., 2011).

A hot flush usually lasts 1–5 min, is typically followed by chills, and is often accompanied by a feeling of irritation, anxiety, or panic, thus affecting quality of life. These symptoms, which have been attributed to increased central sympathetic activation (Freedman, 2001), can be triggered by embarrassment, a sudden change in ambient temperature, stress, and consumption of alcohol, caffeine, or warm beverages (Archer et al., 2011; Sturdee, 2008). The Women's Health Initiative (WHI), an observational study involving 60,000 postmenopausal women, found that hot flushes affected approximately 60% of postmenopausal women (Szmuilowicz et al., 2011). In addition, an estimated 75% of all women experience hot flushes during the transition to menopause (Kronenberg, 1990; Nakano et al., 2012). The median age of menopausal onset is approximately 51 years (Kronenberg, 1990), and the median duration of moderate-to-severe hot flushes is 10.2 years (Avis et al., 2015; Freeman et al., 2011), although they have been observed in some women up to the age of 85 (Vikström et al., 2013). Patients with hot flushes can have measurable changes in the autonomic nervous system, with increased sympathetic and decreased parasympathetic control over their heart rate during hot flushes (Thurston, 2018).

The precise mechanisms that underlie hot flushes in menopausal women are not yet fully understood (Freedman, 2001). Although hot flushes are generally attributed to a decline in estrogen levels during perimenopause, the intensity of vasomotor symptoms (*i.e.*, hot flushes) is not correlated with absolute peripheral estrogen levels, but rather with the rate of change in plasma estrogen concentration, which indirectly affects the thermoregulatory system *via* the hypothalamus (Andrikoula and Prelevic, 2009). This explains why the postmenopausal-like symptoms of abrupt chemotherapy-induced ovarian insufficiency are severe (Adelson et al., 2005; Carpenter et al., 1998). Hot flushes are likely caused by estrogen deficiency–induced changes in hypothalamic thermoregulation, as estrogens can cross the blood-brain-barrier and exert a variety of specific actions in different areas of the brain, particularly the hypothalamus, where estrogens act *via* intracellular estrogen receptors (predominantly ERα, but also ERβ) and *via* non-genomic mechanisms. In addition, estrogens interact with and alter the function of various serotonin (5-HT) receptors (McEwen, 2001) and modulate noradrenergic receptor signaling in the hypothalamus (Ansonoff and Etgen, 2000; Etgen and Karkanias, 1994).

It is generally well accepted that neurotransmitters play a role in the generation of hot flushes, including α_2-adrenergic, serotonergic (Berendsen, 2000), and GABAergic signaling, given that drugs that target these systems have shown clinically beneficial clinical effects in climacteric patients (Nelson et al., 2006). For example, studies have shown an increase in brain norepinephrine levels immediately before the onset of a hot flush, and these levels increased further during the hot flush episodes (Freedman, 2001). When the α_2-agonist clonidine or the α_2-antagonist yohimbine was given to menopausal women a significantly greater number of subjects experienced HF after yohimbine, whereas clonidine reduced the number of hot flushes and increased the temperature threshold to provoke HF (Freedman et al., 1990).

In addition, changes in gonadotropin levels seem to modulate the generation of hot flushes, although whether this role is causal remains unclear. For example, surges in luteinizing hormone (LH) levels are considered to occur secondary to the hypothalamic activation of sympathetic noradrenergic neurons (Kouriefs et al., 2002; Rossmanith and Rubberdt, 2009). Furthermore, results obtained from studies using animal models of temperature dysregulation suggest that estrogen deficiency leads to a decrease in hypothalamic serotonin and norepinephrine concentrations. Postmenopausal women show lower serotonergic activity compared to premenopausal women; after receiving estrogen HRT, their serotoninergic activity becomes partly restored (Blum et al., 1996; Gonzales and Carrillo, 1993; Halbreich et al., 1995). Interestingly, 5-HT_{2A} receptors in the hypothalamus appear to play a role in modulating the development of hot flushes. For example, these receptors are upregulated when estrogen levels decline (Biegon, 1990). Moreover, blocking 5-HT_{2A} receptors in postmenopausal women using the tetracyclic antidepressant mirtazapine reduced the frequency and intensity of hot flushes (Waldinger et al., 2000); however, it is important to note that mirtazapine can also block α_2 adrenergic receptors and H_2 histamine receptors (Anttila and Leinonen, 2001). On the other hand, activating 5-HT_2 receptors using the agonist meta-chlorophenylpiperazine has been shown to induce postmenopausal-like symptoms, including sweating, hot flushes, and palpitations (Berendsen, 2000).

The 5-HT_{1A}, 5-HT_{1D}, and 5-HT_7 receptors have also been shown to play a role in hypothalamic thermoregulation (Hedlund et al., 2003; Naumenko et al., 2011). For example, activating 5-HT_{1A} receptors causes hypothermia, whereas activating 5-HT_{2A} receptors causes hyperthermia (Sturdee, 2008). Moreover, decreased levels of hypothalamic serotonin and norepinephrine in the hypothalamus can be prevented by the serotonin-norepinephrine reuptake inhibitor (SNRI) desvenlafaxine (Deecher et al., 2007); possibly explaining the beneficial effects of selective serotonin reuptake inhibitors (SSRIs) and SNRIs reported in patients with climacteric symptoms (Drewe et al., 2015).

These examples show that temperature regulation - and hot flushes in particular - can be regulated by modulating estrogen receptors, but can also be regulated independently of estrogens. This explains the efficacy of pharmacological treatments that do not affect estrogen levels or estrogen receptor activity, including CRE-mediated and metabolic effects, as discussed below.

Energy metabolism and weight gain in menopause

In addition to their effects on the reproductive system, estrogens exert a variety of antiinflammatory, cytoprotective, and metabolic effects in several non-reproductive organs, as reviewed by (Amantea et al., 2005). Estrogens are particularly important modifiers of systemic energy balance *via* their direct effects on peripheral organs and tissues, and *via* regulatory circuits in the central nervous system (CNS) (Frank et al., 2014; López and Tena-Sempere, 2015; Mauvais-Jarvis et al., 2013).

Estrogen receptors are expressed widely throughout the brain. For example, ERα receptors are expressed in several hypothalamic nuclei, including ventromedial hypothalamus, (VMH), the arcuate nucleus, paraventricular hypothalamus (PVH), and the preoptic area (POA) and lateral area (LHA) of the hypothalamus (Merchenthaler et al., 2004; Osterlund et al., 1998), where they play an important role in maintaining systemic energy homeostasis (López, 2018a). In rats, injecting estradiol into the brain induced strong catabolic effects, increased signaling from the sympathetic nervous system signaling to brown adipose tissue (BAT), and increased thermogenesis (Martínez de Morentin et al., 2014); these effects were mediated by inhibition of the enzyme AMPK (AMP-activated protein kinase) in the hypothalamus (Blanco Martínez de Morentin et al., 2011; López et al., 2016, 2002; Martínez de

Morentin et al., 2014; Merchenthaler et al., 2004; Osterlund et al., 1998). In contrast, activating AMPK in the CNS reversed the effects of estradiol in the hypothalamus (Martínez de Morentin et al., 2014), revealing that AMPK itself is a key regulator - and a potential therapeutic target - of energy metabolism and thermoregulation in the brain.

Binding of norepinephrine to G_S protein−coupled β_3 receptors in BAT adipocytes activates the cyclic AMP protein kinase A (PKA) pathway, driving the lipolysis of free fatty acids and triglycerides, which in turn regulates activity of the mitochondrial carrier protein UPC1 (uncoupling protein 1). Activated UCP1 uncouples the respiratory chain, thus producing heat rather than ATP during lipid breakdown (Cannon and Nedergaard, 2004; Contreras et al., 2015), resulting in thermogenesis in BAT and a rise in Tc. This increase in Tc results in increased energy expenditure and reduced appetite, thereby causing weight loss. In addition to these central effects, estrogens also directly affect BAT by inducing thermogenesis *via* cytoplasmic, non-genomic effects (Frank et al., 2018; Wade and Gray, 1978); for example, estrogens can stimulate β_3 receptor expression and mitochondrial biogenesis (González-García et al., 2017).

In contrast, ERα knockout mice, which serve as a model to study menopause, develop hyperphagia, hypometabolism, and adiposity (Geary et al., 2001; Heine et al., 2000). These observations in animal models translate well into postmenopausal women, as confirmed by the large epidemiological WHI study (Gallicchio et al., 2014). In postmenopausal women, reduced estrogen levels result in altered metabolic control, hyperphagia, decreased energy expenditure, obesity, insulin resistance, and dyslipidemia, all of which are hallmark features of metabolic syndrome (Mauvais-Jarvis et al., 2013). These metabolic effects can be reversed—or at least reduced—by HRT or by directly interfering with the molecular mechanisms that underlie these effects that result from reduced estrogen levels and aging.

Although AMPK plays an important role in regulating metabolism, it has opposite effects depending on its site of activation. Activation of AMPK in the hypothalamus has an overall anabolic effect, whereas activation of AMPK in peripheral tissues induces catabolic effects (Minokoshi et al., 2004, 2002).

In mice, expressing constitutively active AMPK in the hypothalamus increases food intake and body weight, while expressing a dominant negative AMPK in the hypothalamus reduces both food intake and body weight (Minokoshi et al., 2004), likely by inhibiting ghrelin's orexigenic response (López et al., 2008). In the hypothalamus, AMPK is considered the key negative regulator of sympathetically activated thermogenesis, integrating specific peripheral signals mediated by thyroid hormones, estrogens, and metabolites together with different hypothalamic networks and food signals (Blanco Martínez de Morentin et al., 2011; López et al., 2016, 2002; Merchenthaler et al., 2004; Osterlund et al., 1998). Furthermore, nicotine-induced weight loss in rats has been associated with hypothalamic AMPK inactivation and decreased orexigenic signaling. Finally, inhibiting AMPK increases energy expenditure due to increased locomotor activity and increased thermogenesis in BAT (Martínez de Morentin et al., 2012; Seoane-Collazo et al., 2014).

In *peripheral tissues*, AMPK senses a decrease in cellular energy and activates catabolic pathways. In response to an increase in the AMP/ATP ratio, AMPK is activated, inhibiting anabolic pathways and increasing the cellular uptake and turnover of key nutrients such as glucose (Hardie et al., 1999). Thus, peripheral AMPK activation is associated with anti-diabetic effects and either weight loss or weight stabilization. As discussed above, estrogens inhibit the function of AMPK in the hypothalamus, thereby promoting an overall catabolic effect at in the CNS. In the periphery, however, estrogens can regulate insulin sensitivity by acting directly on the pancreas, liver, and lipid metabolism, thus mediating an anabolic effect.

The mechanism underlying these opposite effects of AMPK activation between the CNS and the periphery are poorly understood, but may be attributed in part to tissue-specific differences in AMPK subunits and/or isoforms such as α1, α2, β1, β2, γ1, γ2, and γ3 (Hardie et al., 1998), as summarized in Table 2. For example, in the hypothalamus the α2 subunit plays a more dominant role than the α1 subunit in regulating metabolism (Amato and Man, 2011; Tanida et al., 2013; Viollet et al., 2003a, 2003b), whereas the α1 subunit accounts for 95% of AMPK activity in the liver (Hardie et al., 1998; Wu et al., 2013). In addition, tissue-specific responses of upstream activating kinases such as CaMKK (calmodulin-dependent kinase I kinase), tumor suppressor LKB1 (liver kinase B1), and TAK1 (TGFβ-activated kinase) may contribute to tissue-specific differences in response to metformin, an indirect activator of AMPK. For example, low blood glucose levels were shown to drive phosphorylation of AMPK in rat hypothalamic neurons, similar to the responses observed in liver and muscle cells; however, metformin blocked AMPK activation in hypothalamic neurons under low glucose conditions, which may explain the metformin-induced weight loss (Chau-Van et al., 2007). Similarly, both cannabinoids and ghrelin activate AMPK in the hypothalamus but inhibit AMPK in the liver and adipose tissues, thus leading to overall anabolic effects and weight gain (Kola et al., 2005). Notably, the opposite effects of metformin and cannabinoids on metabolism and body weight have been attributed to AMPK and are independent of estrogen signaling, thus revealing AMPK as a promising target for therapeutic approaches in metabolic diseases.

In skeletal muscle, the AMPKα1 and AMPKα2 subunits are differentially activated (Mounier et al., 2011). For example, AMPKα2 is activated by acute exercise and endurance training, whereas AMPKα1 is activated by low-intensity muscle contraction and oxidative stress; moreover, AMPKα1 plays a critical role in regulating muscle cell size, whereas AMPKα2 is involved in metabolic adaptation in muscle cells (Mounier et al., 2011). Furthermore, the diversity of pharmacological effects is likely due to different subcellular distributions (Miyamoto et al., 2015). For example, membrane-bound AMPK levels (including in the nuclear membrane), are 6-fold higher than AMPK levels in the cytosol, and membrane-associated AMPK is predominantly phosphorylated. This distinct subcellular distribution pattern has also been reported by several groups (Khan and Frigo, 2017; Turnley et al., 1999); the α1 subunit is primarily cytosolic or associated with the plasma membrane in endothelial cells, whereas the α2 subunit appears to be located predominately in the nucleus in pancreatic beta cells, neurons, and skeletal muscle (Steinberg and Kemp, 2009; Turnley et al., 1999).

AMPK and body weight gain during menopause

A nationwide retrospective study in the US found that menopausal women gain body weight (on an average of 2.25 kg and 20% of the patients 4.5 kg and more over a 3-year period (Wing et al., 1991). Such weight gain increases the risk of developing metabolic syndrome and/or diabetes (Szmuilowicz et al., 2009). Weight gain can be partly age-related and partly due to decreased energy expenditure (Polotsky and Polotsky, 2010). On the other hand, a longitudinal MRI study found that total abdominal fat increased in menopause significantly (by an

Table 2
Summary of the dominant AMPK isoforms expressed in various tissues.

Organ	Major AMPK subunit(s)	Reference(s)
Brain		
Hippocampus	α2	(Turnley et al., 1999)
Hypothalamus	α2 (food regulation) α1 (thermoregulation)	(Conde-Sieira et al., 2020)
Heart	Human: α2β2γ1 Rat: α1β1/2γ1	(Wu et al., 2013)
Liver	Human: α1β2γ1 Rat: α1β1γ1 HepG2 cells: α1β1γ1	(Wu et al., 2013)
Skeletal muscle	Human:α2 dominant Rat:higher levels of α2 and β2	(Jessen et al., 2014) (Kopietz et al., 2018)
Adipose tissue	Human: α1β2γ1 3T3L1a cells:α1β1γ1	(Jessen et al., 2014)
Bone tissue	α1 (α2 not present)	(Shah et al., 2010)

average of 26%) despite no change in physical activity or body weight (Franklin et al., 2009). Finally, a study involving 183 postmenopausal women found that the frequency and severity of vasomotor symptoms were significantly correlated with the extent of metabolic changes that occurred during menopause (Lee et al., 2012).

Similar results have been found in animal models. For example, a change in abdominal fat distribution was observed in ovariectomized (OVX) rats, an animal model of menopause (Kapur et al., 2010; Rachon et al., 2008). In this model, ovariectomy increased abdominal fat deposits, body weight, and leptin serum concentration, and these effects were prevented by treating the OVX rats with estradiol or estradiol benzoate. Another study involving OVX rats found that body weight increased at a significantly higher rate than in sham-operated rats fed the same diet, whereas estradiol treatment prevented this difference between OVX and sham-operated animals (Martínez de Morentin et al., 2014).

A growing body of evidence suggests that AMPK signaling plays a significant role in menopause-induced weight gain. For example, intracerebral injections of the AMPK inhibitor dorsomorphin (also known as compound C) reversibly inhibited weight gain in OVX rats (Tsai et al., 2010). Furthermore, oral administration of metformin reduced weight gain and early metabolic changes in OVX rats (Barthem et al., 2019). In obese, insulin-resistant male *ob/ob* mice—which are unable to produce leptin—administration of the *Cimicifuga racemosa* extract Ze 450 both reduced weight gain, improved glucose tolerance, and reduced insulin resistance in a dose-dependent manner (Moser et al., 2014). *In vitro* experiments in HepaRG cells (an human hepatic cell line) showed dose-dependent AMPK activation by Ze 450 and some of its constituents, suggesting that the *in vivo* effects of Ze 450 in male *ob/ob* mice were due—at least in part—to activation of peripheral AMPK (Moser et al., 2014).

Recently, Yuan et al. reported that 23-*epi*-26-deoxyactein, a major constituent of *Cimicifuga racemosa*, inhibits the formation of adipocytes from stem cells (*i.e.*, adipogenesis) in the 3T3-L1 preadipocyte cell line (Yuan et al., 2020). This effect was accompanied by significant increases in SIRT1 protein levels, expression of adipose triglyceride lipase, and AMPK activation. Furthermore, the authors found that administering 5 mg/kg/day 23-*epi*-26-deoxyactein to wild-type C57BL/6 mice fed a high-fat diet for 12 weeks significantly reduced weight gain, fat mass, and liver mass compared to mice that were fed the high-fat diet but were not given 23-*epi*-26-deoxyactein (Yuan et al., 2020).

With respect to the correlation between an increased risk of developing diabetes and climacteric symptoms, conflicting results have been reported. For example, a study involving 328 women who underwent natural menopause found no increased risk of diabetes compared to 708 premenopausal age-matched women (Kim et al., 2011). On the other hand, a large study involving 150,000 postmenopausal women examined the association between diabetes and hot flushes and found that an increasing severity and duration of hot flushes was associated with an increased risk of developing diabetes, independent of obesity; moreover, this risk was even more higher among women who also reported experiencing night sweats (Vara-Ciruelos et al., 2018). Although no causal link between hot flushes and an increased risk of diabetes has been established, it is interesting to note that HRT for treating menopausal symptoms can often also improve diabetes (Szmuilowicz et al., 2009).

As discussed above, the enzyme AMPK plays a critical role in regulating metabolism at two levels. In the CNS, hypothalamic AMPK is considered the key negative regulator of sympathetically activated thermogenesis, systemic metabolism, and food signals (Blanco Martínez de Morentin et al., 2011; López et al., 2016, 2002; Merchenthaler et al., 2004; Osterlund et al., 1998). In cells in the periphery, AMPK senses a decrease in cellular energy; in response to an increased AMP/ATP ratio, AMPK is activated, thereby driving catabolic pathways, inhibiting anabolic reactions, and increasing the cellular uptake of glucose and other nutrients, thereby improving insulin sensitivity and glucose homeostasis (Hardie et al., 1999). Thanks in part to numerous clinical studies showing the efficacy of the indirect AMPK activator metformin in patients with type 2 diabetes, this drug is now recommended by the American Diabetes Association as first-line treatment for managing elevated blood glucose in adults with type 2 diabetes mellitus (ADM, 2019). Taken together, these findings indicate that pharmacologically targeting AMPK-dependent metabolic signaling is both safe and effective for treating menopause- and/or age-related weight gain and diabetes, independent of estrogen receptor regulation.

AMPK and cardiovascular risks in menopause

In addition to causing metabolic changes such as altered body fat distribution and dyslipidemia, several studies have shown that menopause is a general risk factor for cardiovascular disease, as menopause can increase blood pressure and sympathetic tone and cause endothelial dysfunction and vascular inflammation, for example as demonstrated in the Framingham Heart Study (Rosano et al., 2007). In a large cohort consisting of Dutch and Swedish women aged 46–76 years with no symptoms of hot flushes prior to menopause, nearly 11,000 women were followed for up to 10.3 years; the study found that women with night sweats during menopause had a moderately increased risk of coronary heart disease (CHD), and this increased risk could not be fully explained by typical CHD risk factors (Gast et al., 2011). Moreover, the observational WHI study (Szmuilowicz et al., 2011) found that hot flushes were associated with a decreased cardiovascular risk, but only when the hot flushes were limited to the onset of menopause; women with persistent or late-onset hot flushes had a significantly increased hazard ratio for CHD, stroke, total cardiovascular events, and all-cause mortality (Szmuilowicz et al., 2011). In the US-based prospective Study of Women's Health Across the Nation (SWAN) involving the transition to menopause, cardiovascular risk factors were compared between 1054 women who reached menopause and 380 premenopausal women, revealing differences in total cholesterol, low-density lipoprotein (LDL) cholesterol, and apolipoprotein B levels; other risk factors were consistent with aging (Matthews et al., 2009a). More recently, a 3-month study involving 15 women with menopausal symptoms and 15 premenopausal controls found that the women in the menopausal group had higher blood pressure, inflammatory cytokine levels, and oxidative stress compared the control group (Vassalle et al., 2011).

These findings have been supported by animal studies. For example, studies involving obese insulin-resistant OVX rats showed that either estrogen deprivation or obesity alone altered metabolic parameters, cardiac function, and mitochondrial function; moreover, obesity combined with insulin resistance accelerated and aggravated these cardiometabolic changes in estrogen-deficient rats (Sivasinprasasn et al., 2015). Estrogen-related receptor alpha (ERRα) has also been shown to play an important role in regulating energy homeostasis and mitochondrial biogenesis in various different cardiac functions related to myocardial energy, as reviewed by Ranhotra (Ranhotra, 2015).

The putative beneficial effects of targeting AMPK signaling for cardiac function have been investigated in several *in vitro* and *in vivo* animal studies. For example, cardiac tissue samples obtained from patients with congestive heart failure and from animals with heart failure showed that AMPKα2 regulates the expression of ERRα and downstream mitochondrial enzymes (Hu et al., 2011). Recently, Bussey et al. studied the function of hearts isolated from obese Zucker rats and found that reduced AMPK signaling was related to a decreased β-adrenergic response (Bussey et al., 2018). Moreover, several studies suggest that activating AMPK can inhibit the processes leading to cardiac remodeling and hypertrophy, inflammatory responses, and fibrosis (Feng et al., 2018). For example, AMPK activation was shown to prevent myocardial hypertrophy in wild-type mice but not in mice lacking AMPKα2 (Gelinas et al., 2018). In a separate study, application of metformin either before inducing experimental transient ischemia or at the time of reperfusion reduced myocardial injury in both nondiabetic and diabetic (*db/db*) mice (Calvert et al., 2008). In addition, a large body of evidence supports the notion that AMPK signaling have a protective role during ischemia

and ischemia-reperfusion (Qi and Young, 2015). This notion is supported further by a report by Zaha et al., who measured hearts isolated from wild-type mice and from mice expressing an inactive mutant form of AMPKα2; the authors found that intrinsic AMPK activation was required for preventing irreversible mitochondrial damage and myocardial injury during both ischemia and reperfusion (Zaha et al., 2016).

During menopause, women who experience hot flushes also tend to experience other symptoms such as a change in libido, dyspareunia, depression, musculoskeletal pain, vaginal pruritus and dryness, inflammation, and accelerated osteoporosis due to increased bone turnover. These symptoms can be directly related to estrogen deficiency or they can be indirect age-related complaints that become aggravated by the decrease in estrogen (Burger et al., 2002; Crandall et al., 2011; NAMS, 2004).

AMPK effects on the CNS: mood disorders and cognitive impairment

Two scales commonly used to assess the severity of menopausal symptoms, namely the Menopause Rating Scale (MRS) (Heinemann et al., 2003) and the Kupperman Menopausal Index (KMI) (Drewe et al., 2013) include psychological symptoms; specifically the MRS includes "depressed", "irritable", "anxious", and "exhausted", while the KMI includes "nervousness" and "melancholia". Moreover, these symptoms usually improve with treatment (see for example (Drewe et al., 2013; Schneider et al., 1977; Whooley et al., 2000)). However, signs of depression in menopausal women varied a lot in older studies. There are reports that although not present in every woman at the time of menopause, many women develop such symptoms "especially during the perimenopausal period" (Richardson and Robinson, 2000). In addition, the SWAN study involving 221 African-American and Caucasian women found that perimenopausal and early postmenopausal women were 2–4 times more likely to experience a major depressive episode when they were peri-menopausal or early post-menopausal (Bromberger et al., 2011).

Estrogens can affect mood *via* several pathways, including increased serotonergic activity, modulation of presynaptic and/or postsynaptic serotonin receptors, and neurotrophic processes such as promoting neuroplasticity or neurogenesis. Many of these effects appear to be mediated *via* the ERβ estrogen receptor (Osterlund, 2010). Using a rat model of middle cerebral artery occlusion, Su and colleagues found that estrogen therapy improved post-stroke depression (measured using the forced swimming test) and other neurological symptoms in OVX rats. In the hippocampus, estrogen-induced expression of brain-derived neurotrophic factor (BDNF) was reported (Su et al., 2016). BDNF serves as an established biomarker for depression (Bocchio-Chiavetto et al., 2010; Peng et al., 2018). These effects of estrogen on BDNF expression may be due to estrogen binding to the estrogen response element in the BDNF gene; in OVX rats 28 weeks after surgery, BDNF mRNA levels were significantly reduced in nearly all hippocampal layers and in the cortex (Kino et al., 2010; Singh et al., 1995).

Interestingly, exercise also ameliorates depression-like behavior and increases hippocampal BDNF levels in OVX rats (Lu et al., 2014). In addition, applying electrical stimuli to C2C12 cells (a mouse skeletal myoblast cell line) causes contractions and increased BDNF mRNA levels, as well as an AMPK-dependent increase in fatty acid oxidation (Matthews et al., 2009b).

In an unpredictable chronic mild stress model of depression, mice showed suppressed body weight gain, increased anxiety and increased immobility in forced swimming and tail suspension test, and this was associated with AMPK inactivation in cortical brain areas (Zhu et al., 2014). On the other hand, exercise could has been shown to activate brain AMPK/PGC-1α/BDNF pathway in different animal models (Azimi et al., 2018; Chrysostomou et al., 2016). Along these same lines, exercise has been shown to ameliorate depression-like behavior and increase hippocampal BDNF levels in OVX rats (Lu et al., 2014). In addition to being expressed in the brain, BDNF is also expressed in plasma and in peripheral tissues, particularly skeletal muscle; moreover, BDNF increases fat oxidation by activating AMPK (Matthews et al., 2009b; Pedersen et al., 2009).

With respect to cognition, this can be assessed by measuring attention and working memory, verbal learning and verbal memory, and fine motor speed. Weber et al. found that cognition is decreased in perimenopause, particularly in the first year after menopause relative to premenopause and late postmenopause; moreover, these cognitive domains are supported by the hippocampus and prefrontal cortex, brain structures that are targets for estrogens (Weber et al., 2013).

Interestingly, Keenan and colleagues compared 9 menopausal women taking HRT and 10 menopausal women who were not taking HRT and found that the non-treated group performed worse than the HRT group at several tasks in a battery of neuropsychological tests (Keenan et al., 2001). However, age may have been a confounding factor. this was studied in an electrophysiological study in postmenopausal women with age related cognitive decline and age-match normal postmenopausal control. Using P300 latency for stimulus classification speed and P300 amplitude as a measure of attentional resource allocation when memory updating is engaged, both groups were evaluated for event-related potentials *i.e.*, before and after therapy. Before estrogen treatment, patients showed a lengthening of P300 latency and an attenuation of P300 amplitudes as compared with normal controls indicating an advance. After estrogen treatment, a significant improvement and normalization of information processing as indexed by P300 were observed with electrophysiological markers of cognitive processes (Anderer et al., 2005).

These results are supported by animal studies. For example, in OVX rats the sensitivity of the hippocampus to estradiol-induced increases in CA1 apical dendritic spine density was progressively reduced in relation to the time since OVX, reflecting a decrease in synaptic plasticity and—consequently—cognitive ability (McLaughlin et al., 2008). In a recent review, this regulation of synaptic plasticity by estrogen was emphasized in the context of affecting cognition (Arevalo et al., 2015). Previously, Maki and Dumas reviewed various neuroimaging and psychopharmacological studies showing that estrogen can improve memory by interacting with the cholinergic and serotonergic systems in the hippocampus and frontal cortex (Maki and Dumas, 2009).

Recently, regular prolonged training in mice was shown to reduce chronic stress–induced memory deficits; this effect was mediated by AMPK-mediated induction of BDNF. Moreover, these effects were increased in a dose-dependent manner by treatment with the AMP analog 5-aminoimidazole-4-carbox-amide-1-β-D-ribofuranoside (AICAR) and blocked by treatment with the AMPK inhibitor compound C (Kim and Leem, 2016). Further evidence supporting the role of AMPK in cognition was obtained recently by Bang et al., who showed that treating aged mice with the metformin derivative HL271 improved cognitive performance in aged mice, and this improvement was correlated with increased levels of phosphorylated AMPK in the brain (Bang et al., 2018).

Overall, these results obtained using animal models and in patients confirm the significant role that decreased estrogen levels play in the development of mood disorders and cognitive impairments. However, aging itself can have confounding effects with respect to impaired brain function and neural homeostasis, and AMPK-regulated metabolism and neurotrophic growth factor signaling are major determinants of neural plasticity and brain homeostasis, with promising new implications in the future therapy for neurological and neuropsychiatric diseases.

AMPK effects on bone: osteoporosis

With increasing age, both men and women have an increasing risk of developing osteoporosis. However, before the onset of menopause, women are partially protected from osteoporosis by relatively high levels of estrogens. After the onset of menopause—particularly in the case of early menopause (*i.e.*, under the age of 45), both age and reduced estrogen are important risk factors for accelerated osteoporosis and

increased susceptibility to bone fractures (Worley, 1981).

Bone remodeling is an energy-intensive process regulated primarily by AMPK based on available nutrient reserves (Jeyabalan et al., 2012). The effect of AMPK activation on bone formation has been studied in cultured primary rat osteoblasts, in which both AICAR and metformin dose-dependently increased trabecular bone nodule formation; in contrast, this process was inhibited by compound C (Shah et al., 2010). These effects were supported by *in vivo* studies with 4-month-old transgenic AMPKα1 knockout mice, in which micro-computed tomography showed smaller cortical and trabecular bone compartments compared to age-matched wild-type mice (Shah et al., 2010). In addition, genetic deletion of AMPKβ subunits in mice impaired bone formation without affecting osteoclast function, leading to net bone loss (Quinn et al., 2010; Shah et al., 2010). In contrast, metformin was shown to upregulate the expression of osteoprotegerin (also known as osteoclastogenesis inhibitory factor) in OVX rats, inducing osteoblasts and inhibiting osteoclast differentiation (Jiating et al., 2019; Mai et al., 2011). Likewise, the flavonoid hispidulin has been shown to have anti-osteoporotic effects in OVX mice by activating AMPK signaling (Zhou et al., 2014).

These observations in animal models have translated into clinically relevant benefits. For example, a Danish case-control study involving approximately 125,000 diabetic patients with fractures and approximately 375,000 control diabetics found that taking metformin was associated with a significantly decreased risk of bone fracture (Vestergaard et al., 2005). Similarly, a historical cohort study involving nearly 2000 diabetic patients showed that the risk of developing fractures was significantly reduced (with an age-adjusted hazard ratio of 0.7; 95% CI: 0.6–0.96) by treatment with biguanide compounds such as metformin (Melton et al., 2008). Recently, a meta-analysis of 6 clinical studies showed an inverse correlation between metformin use and the prevalence of bone fractures, with a relative risk of 0.83 (95% CI: 0.72–0.93) (Salari-Moghaddam et al., 2019). Despite these findings, however, little is known regarding the effects of metformin on bone metabolism and the risk of osteoporosis risk in non-diabetics.

Cimicifuga racemosa extracts (CREs)

The active ethanolic Cimicifuga dry extract Ze 450 (ethanol: 60% V/V) is generated from the rhizomes and roots of *Cimicifuga racemosa*, with a drug-extract ratio (DER) of 4.5–8.5. Other CREs are also available for clinical use and include: BNO 1055 (ethanol 58% V/V) with a DER of 5 - 10:1 and the isopropanolic dry extract iCR (isopropanol 40% V/V) with a DER of 6 −11:1.

Several randomized controlled trials (RCTs) and non-interventional observational studies have been published showing a clinically significant effect in controlling climacteric complaints in women: with an isopropanolic extract (Bai et al., 2007; Liske et al., 2002; Nappi et al., 2005; Osmers et al., 2005; Ross, 2012), with the ethanolic extract BNO 1055 (Aly, 2009; Wuttke et al., 2006, 2003b), and with the ethanolic extract Ze 450 (Drewe et al., 2013; Friederichsen et al., 2020; Lopatka et al., 2007; Schellenberg et al., 2012).

Based on numerous *in vitro* and *in vivo* animal studies, as well as the clinical studies described above, these extracts have been granted a "well-established use" status by the European Medicines Agency (EMA, 2017, 2018). Given the comparable efficacy and toxicity of these extracts, we consider that the statements below are valid for all CREs listed in the EMA's monograph (EMA, 2017, 2018).

CRE constituents

As with all herbal drug mixtures, *Cimicifuga racemosa* extracts consist of a large number of structurally distinct chemical compounds. Over the past 40 years, a wide range of compounds have been identified and isolated from aqueous-alcoholic CREs. The main constituents in CREs are triterpene glycosides, a complex mixture from which over 200 compounds have been structurally identified as belonging primarily to the cycloartane-type family of triterpenoids (Li and Yu, 2006). The principal triterpene glycosides appear to be cimicifugoside, 23-*epi*-26--deoxyactein, and cimiracemosides A, C, and F. These triterpene glycosides are either 3-*O*-xylosides or 3-*O*-arabinosides of cycloartane-type aglycones. A highly comprehensive review of the constituents found in CREs has been published previously (Li and Yu, 2006). Other constituents are derivatives of organic acids, such as fukiic and piscidic acid esters, especially fukinolic acid, aromatic acids such as caffeic, ferulic and isoferulic acids, and various phenyl propanoid esters such as methyl caffeate, petasiphenol, cimiciphenol and cimiracemates A-D. Further constituents include organic acids, especially butyric acid, gallic acid, palmitic acid and salicylic acid, as well as tannin, resin and essential oils.

Of these compounds, ferulic acid (Chen et al., 2019; Luna-Vital et al., 2020), 23-*epi*-26-deoxyactein and cimiracemoside C (Moser et al., 2014; Yuan et al., 2020), caffeic acid (Tyszka-Czochara et al., 2017), caffeic phenyl ester (Ferreira et al., 2019; Li et al., 2019), salicylic acid (Hawley et al., 2012), gallic acid (Doan et al., 2015) as well as the whole ethanolic CRE Ze 450 (Moser et al., 2014) were shown to activate AMPK in different *in vitro* assays.

Pharmacokinetics of CRE constituents. Relevant absorption of main constituents of CREs following oral administration is required before exerting systemic effects. Enteral absorption of the isolated triterpene glycoside 23-*epi*-26-deoxyactein was confirmed *in vitro* by measuring its penetration through Caco-2 cell monolayers, revealing high permeability. When injected *in situ* into various isolated segments of rat intestine, a good absorption was observed from duodenum, jejunum, ileum, and colon, with the highest and lowest absorption rates measured in the duodenum and colon, respectively; however, when adjusted for segment length and transit time through each segment, the jejunum, ileum, and colon appeared to have comparable absorption (Disch et al., 2017).

The pharmacokinetics of 23-*epi*-26-deoxyactein was also assessed in a clinical study in which women received a standardized oral dose of an ethanolic CRE, revealing that the absorption of 23-*epi*-26-deoxyactein was non-linear with an over-proportional area under the curve (AUC) at higher doses, thus suggesting a saturable elimination process (van Breemen et al., 2010). Accordingly, the authors also found that the terminal half-life of 23-*epi*-26-deoxyactein increased with increasing doses, from 2.1 h at a dose of 1.4 mg to 3.0 h at a dose of 5.6 mg. In addition, incubating 23-*epi*-26-deoxyactein with human hepatocytes did not reveal either phase I or phase II metabolism such as oxidation, conjugation, or de-glycosylation; however, a small degree of degradation was observed in artificial human gastric fluid (van Breemen et al., 2010).

Pharmacological effects of CRE constituents. The pharmacological effects of various CREs and their isolated constituents have been investigated *in vitro, in vivo*, and in clinical studies. For example, Rachon and colleagues studied the effects of the ethanolic CRE extract BNO 1055 and estradiol benzoate on body weight gain, intra-abdominal fat accumulation, plasma lipid levels, and glucose tolerance in OVX Sprague-Dawley rats. The authors found that both animals treated with BNO 1055 and animals treated with estradiol gained significantly less weight and showed significantly less intra-abdominal fat accumulation compared to untreated OVX rats. In contrast, estradiol treatment—but not BNO 1055 treatment—significantly reduced the AUC in the oral glucose tolerance test (OGTT) compared to control-treated OVX rats (Rachon et al., 2008).

In extension to the above study in OVX female rats, the effects of the ethanolic CRE extract Ze 450 on metabolism were evaluated in male obese and diabetic *ob/ob* mice (Moser et al., 2014). When administered orally for 7 days, Ze 450 significantly reduced both daily weight gain and cumulative weight gain. During OGTT, the Ze 450–treated mice had significantly decreased post-stimulated insulin and HOMA-IR (homeostasis model assessment of insulin resistance) compared to

metformin-treated and placebo-treated controls. Moreover, some of the isolated constituents of Ze 450 (*e.g.*, protopine, cimiracemoside, and 23-*epi*-26-deoxyactein) caused a dose-dependent increase in AMPK activation in cultured HepaRG cells, supporting the notion that AMPK activation is the mechanism underlying the anti-diabetic and body weight effects observed *in vivo* in the *ob/ob* mice (Moser et al., 2014). Recently, Yuan et al. reported that 23-*epi*-26-deoxyactein inhibited adipogenesis in 3T3-L1 preadipocytes cells (Yuan et al., 2020). These changes were linked to significant increases in SIRT1 protein levels and adipose triglyceride lipase expression, as well as increased AMPK activation. Furthermore, the authors found that treating 5-week-old male C57BL/6 mice fed a high-fat diet with 5 mg/kg/day 23-epi-26-deoxyactein significantly reduced weight gain, fat mass, and liver mass compared to untreated mice.

Clinical studies involving CREs

Although no studies have shown that CRE constituents can directly reach the brain, indirect evidence of their effects in the brain have been reported using neuroimaging. For example, CRE was given to postmenopausal women at a dose of 40 mg/day for 12 weeks, and effects were measured in various brain regions using positron emission tomography (PET); specifically, significant increases in μ-opioid receptor binding were measured in brain regions involved in emotional and cognitive function, including the posterior and subgenual cingulate cortex, the temporal and orbitofrontal cortex, thalamus, and *nucleus accumbens*, with increases ranging from 10% to 61% across brain regions involved in emotional and cognitive function (Reame et al., 2008). In another study involving 20 healthy volunteers, the effects of CRE on the stress response were measured in an experimental psychological stress test (Nadaoka et al., 2012). The authors found that CRE reduced subjective stress scores and reduced salivary levels of chromogranin-A , a marker of psychosocial stress (Kanamaru et al., 2006), compared to placebo; in addition, CRE reversed the reduction in α-bands measured on the electroencephalogram (Nadaoka et al., 2012). However, the authors could not rule out the possibility of indirect effects of CRE mediated by afferent nervous signals arising from the periphery.

These human studies have been corroborated by a study involving Sprague-Dawley rats in which the rats were divided in four groups (sham operation plus vehicle, OVX plus vehicle, OVX plus estradiol, and OVX plus CRE) and treated for 4 weeks. Immunohistochemistry revealed reduced density of α_1-adrenergic receptors and ERRα−positive neurons in vehicle-treated OVX rats compared to the sham group. Moreover, these changes were normalized in the estradiol-treated OVX rats. Finally, co-expression of α_1-adrenergic receptors and ERRα was measured in the preoptic area of the hypothalamus in the CRE-treated OVX rats (Wang et al., 2015).

Taken together, although evidence of direct brain penetration is lacking, several studies have provided compelling indirect evidence that CREs exert effects in the CNS.

An extended view of the mechanism of action of CRE

Although not yet fully understood, the current consensus regarding the mechanism of action of CRE is based on the hypothesis that changes in the α-adrenergic and serotonergic signaling pathways secondary to the loss of estrogen underlie the development of hot flushes in menopausal women. Thus, CRE binding to the 5-HT_{1A}, 5-HT_{1D}, and 5-HT_7 serotonin receptors (Burdette et al., 2003); and in particular of the CRE constituent N_ω-methylserotonin (Powell et al., 2008) was demonstrated. This findings suggests that the effect of CRE on hot flushes may be mediated—at least in part—*via* these receptors. However, in OVX rats oral N_ω-methylserotonin blunted hot flush–like symptoms to a similar extent as estrogen implants but had no effect on OVX-induced weight gain or mood-related behaviors (Weiser et al., 2013). In addition, estrogen has been shown to increase the density of 5-HT_{2A} receptors in the *nucleus accumbens*, suggesting that a reduction in estrogen can affect mood and may explain the onset of depressive symptoms during menopause (Fink and Sumner, 1996; Fink et al., 1996). Nevertheless, these putative mechanisms do not necessarily explain all of the effects mediated by CRE, indicating that further studies are clearly warranted.

Importantly, the complementary view of AMPK activation as a central target of CREs is not inconsistent with previous results; rather, it extends the current hypothesis and explains thereby other potential applications of CREs beyond the symptomatic treatment of climacteric complaints. This is particularly relevant when integrating the metabolic effects of CRE on non-vasomotor symptoms in the overall mechanism of action.

CREs and oxidative stress

Oxidative stress, defined as the excessive formation of reactive oxygen species (ROS) as part of decline in mitochondrial function, is one of the pathogenetic factors associated with age-related diseases (Bautista-Nino et al., 2016; Blake and Trounce, 2014), diabetic complications (Araki and Nishikawa, 2010), ischemia-reperfusion—induced toxicity in various tissues (Lin et al., 2017; Qi and Young, 2015; Zaha et al., 2016), and mood disorders (Culmsee et al., 2018; Manji et al., 2012). As complex I and III of the respiratory chain are the source of superoxides giving rise to ROS, inhibition of the respiratory chain results in a simultaneous decrease in ATP and ROS formation thereby attenuating its toxic effects of ROS (Li et al., 2013). Interestingly, metformin (an indirect activator of AMPK) inhibits respiratory chain activity in a dose-dependent manner, causing an increase in aerobic glycolysis (Andrzejewski et al., 2014). Previously, Burdette et al. found that six constituents in CRE protect against menadione-induced DNA damage in cultured S30 breast cancer cells by scavenging ROS (Burdette et al., 2002). After performing bioassay-directed isolation, the authors ranked these constituents based on their activity as follows: methyl caffeate > caffeic acid > ferulic acid > cimiracemate *A* > cimiracemate *B* > fukinolic acid. Furthermore, 23-*epi*-26-deoxyactein protected osteoblastic MC3T3-E1 cells from antimycin A-induced toxicity, which is characterized by dissipation of mitochondrial membrane potential, complex IV inactivation, ATP loss, intracellular calcium ($[Ca^{2+}]_i$) elevation and lethal oxidative stress (Choi, 2013a). Similar protective effects of 23-*epi*-26-deoxyactein were observed by this group using different toxicants: 2,3,7,8-tetrachlorodibenzo-p-dioxin (Suh et al., 2018) and methylglyoxal (Suh et al., 2017b). Finally, it protects pancreatic beta-cells against methylglyoxal-induced oxidative cell damage by the upregulation of mitochondrial biogenesis (Suh et al., 2017a).

These observed cytoprotective effects of CRE against oxidative stress are only partially explained by the mentioned antioxidative capacity of some of the CRE components but largely depend on the interference with mitochondrial metabolism and mitochondrial ROS formation (Rabenau et al., 2021). As shown recently in neuronal and non-neuronal cells, a dose-dependent shift from mitochondrial respiration to glycolysis was detected for Ze 450 *in vitro* (Rabenau et al., 2019), and this was attributed to direct effects of the CRE on the mitochondrial respiratory chain (Rabenau et al., 2021). The CRE reduced the rate of mitochondrial oxygen consumption and increased the extracellular acidification rate, and prevented ROS formation while keeping mitochondrial morphology intact after exposure of the cultured cells to oxidative stress (Rabenau et al., 2021). These data revealed metabolic effects of Ze 450 that preserved mitochondrial integrity and function, and attenuated adverse effects of oxidative dysregulation that are hallmarks of aging, including postmenopausal changes. Notably, in neuronal and non-neuronal cells Ze 450 induced a metabolic shift from mitochondrial respiration to glycolysis very similar to, yet more potent than effects observed with metformin. Moreover, all of these effects were independent of estrogen receptor signaling, confirming that CRE protects against oxidative cell damage *via* metabolic effects. Most importantly, these *in vitro* findings using acute models of oxidative cell damage were recently translated into increased longevity in *Caenorhabditis elegans*. When treated with

CRE, *C. elegans* was protected from mitochondrial toxins in a model of acute toxicity, showed expansion of life span and preserved neurological function, and these effects were again exceeding the effects of metformin (Rabenau et al., 2021). These findings provide compelling evidence that the metabolic effects mediated by CRE may serve as a promising therapeutic approach to mitigate age-related mitochondrial impairments and other cellular and organ dysfunctions related to oxidative stress.

CREs and osteoporosis

The isopropanolic CRE iCR has been shown to induce the expression of osteoprotegerin in human osteoblasts and upregulate the osteoblastic markers bone-specific alkaline phosphatase and osteocalcin (Viereck et al., 2005). Moreover, the triterpenoid glycoside 25-acetylcimigenol xylopyranoside was shown to block *in vitro* osteoclastogenesis bone marrow macrophages induced by either RANKL (receptor activator of NF-κB ligand) or TNFα (tumor necrosis factor α) (Qiu et al., 2007). Isolated 23-epi-26-deoxyactein has also been shown to stimulate osteoblast function and inhibit bone-resorbing mediators in murine osteoblastic MC3T3-E1 cells (Choi, 2011, 2013a); in addition, 23-epi-26-deoxyactein caused significant cell growth, decreased ROS formation, and decreased osteoclast differentiation−inducing factors such as TNFα, IL-6, and RANKL (Choi, 2013b; Lee and Choi, 2014).

Furthermore, the mechanism underlying CRE's anti-osteoporotic effects was studied in both OVX rats and OVX mice. In OVX rats, administration of an isopropanolic CRE for 7 weeks resulted in a reduced urinary concentration of the bone resorption markers pyridinoline and deoxypyridinoline compared to control. Further, OVX rats lost significant amounts of trabecular bone mineral density (BMD), and this was prevented by estradiol treatment and partly by the triterpene-saponin CRE fraction but not by the polar fraction (Seidlova-Wuttke et al., 2012). However, another study found that although estradiol increased volumetric BMD and peripheral quantitative computed tomography compared to controls, CRE showed no significant effects on BMD (Nisslein and Freudenstein, 2003). On the other hand, Cui et al. found that treating OVX rats for 12 weeks with an isopropanolic CRE had a similar effect on BMD and bone structure as estradiol (Cui et al., 2013).

In OVX mice, three isolated cycloartane-type triterpenoids (cimicidol-3-O-β-D-xyloside, cimicidanol-3-O-β-D-xyloside, and acetylacteol-3-O-β-D-xyloside) inhibited osteoclastic bone resorption by suppressing both osteoclast-like cell formation and osteoclast bone-resorbing capacity, thus showed a significant protective effect on BMD (Li et al., 2007).

In a double-blind placebo-controlled study, 62 postmenopausal women were given either the CRE BNO 1055 or conjugated estrogens. Both active treatments were equipotent with respect to menopausal symptoms and bone markers of bone formation such as alkaline phosphatase (Wuttke et al., 2003b).

These data obtained in cell cultures, animal models, and clinical studies demonstrate that CRE and its specific triterpenoid constituents are at least as potent as estrogen in preventing osteoporosis. Future studies are therefore warranted in order to identify the specific components in CRE that prevent osteoporosis and to further elucidate the molecular mechanisms that underlie these estrogen receptor−independent effect. The ability to treat—or even prevent—osteoporosis is a growing medical need in our aging society, particularly among postmenopausal women. Therefore, the therapeutic effects of CRE may be extended to include new complementary new therapeutic strategies for treating osteoporosis.

CREs and cell proliferation/cancer

It may seem tempting to assume that the reduction in estrogen production can best be treated by substitution with estrogen, and indeed this was the case for years, until evidence emerged indicating that this can cause severe adverse effects. For example, the large WHI study found that women who were treated with estrogen either alone or in combination with progestin had a significantly higher rate of breast cancer compared to women who received placebo (Chlebowski et al., 2003, 2019). Therefore, the question is of high relevance, as to whether CREs have also estrogenic effects and carry the same risk of tumor induction.

In early studies, binding of different CREs or their components to cytosolic estrogen binding site was reported (Düker et al., 1991; Jarry et al., 1985, 2003). In OVX rats, binding of CRE BNO 1055 showed different estrogenic effects in the brain, uterus and bone, and since tissue pharmacological profile could not be explained by an estrogenic action of CRE a selective estrogen receptor modulator (SERM) mechanism of action was postulated (Jarry et al., 1999, 2002; Liu et al., 2001b; Seidlová-Wuttke et al., 2003; Wuttke et al., 2003a, 2000). Interestingly, the authors did not observe an estrogenic effect on the uterus of the OVX rats. The bone effects discussed above, however, could potentially be alternatively explained by AMPK mediated effects of Cimicifuga.

One of the classical SERM compounds is tamoxifen. Due to its estrogen antagonistic effect in the brain, it aggravates hot flushes in postmenopausal women, in contrast to CREs (Mourits et al., 2001; Powles et al., 1994). Later on, Wuttke and coworkers distanced themselves from the view that CREs are herbal SERMs (Seidlova-Wuttke, 2010).

Likewise, more recent studies found no evidence that CREs bind to ERα or ERβ receptors, nor did they find any proliferative effects of CREs in a wide range of estrogen-sensitive tumor cells (MCF-7 and drug resistant R-MCF-7, MDA-MB-231; Ishikawa cells, T-47D and 293T cells; LnCaP, PC-3 and DU 145 prostate cancer cells)(Amato et al., 2002; Liu et al., 2001a; Lupu et al., 2003; Nelson et al., 2006; Wuttke et al., 2014). Moreover, several studies also found no evidence of antiproliferative effects after CRE administration (Al-Akoum et al., 2007; Bodinet and Freudenstein, 2002, 2004; Fang et al., 2011; Garita-Hernandez et al., 2006; Gaube et al., 2007; Hostanska et al., 2004a, 2005, 2007; Jarry et al., 2005; Rice et al., 2007; Seidlova-Wuttke et al., 2006; Zierau et al., 2002).

Gaube et al. studied the effect of a lipophilic CRE and purified cycloartane-type triterpenoids on ER-positive MCF-7 cells and found that both treatments inhibited cell proliferation (Gaube et al., 2007). In addition, gene expression profiling identified 411 genes sensitive to treatment of CRE and estradiol including genes involved in anti-proliferation and apoptosis induction, as well as genes involved in several stress response pathways. A similar expression profile was induced by treating the cells with the cycloartane-type triterpene glycoside actein and the triterpene aglycons. Similar results were obtained by treating the human breast cancer cells MDA-MB-453 with a methanolic CRE (Einbond et al., 2007).

Notably, the inhibitory effects of the ethanolic CRE Ze 450 on estrogen-insensitive tumor cells (*e.g.*, ER-negative MCF-7 clone, T-47D cells, and HEK293T cells) were independent of both estrogen receptor and progesterone receptor activation (Garita-Hernandez et al., 2006). In estrogen-positive MCF-7 breast cancer cells, two major fractions of an isopropanolic CRE, namely the triterpene saponins and the cinnamic ester fraction, induced a comparable apoptotic effect, whereas the cinnamic ester fraction was more effective at inhibiting cell proliferation (Hostanska et al., 2004b). Interestingly, CREs were shown to have an anti-proliferative effect in both an ER-negative Her2-overexpressing human breast cancer cell line (MDA-MB-453) and an ER-positive Her2-overexpressing cell line (MCF-7); in addition to the CRE, the constituent triterpene saponins 25-acetyl-7,8-didehydrocimigenol 3-*O*-β-D-xylopyranoside, cimigenol 3-*O*-β-D-xyloside, and actein (β-D-xylopyranoside) had a significant inhibitory on cell proliferation (Einbond et al., 2006, 2008). Similar results were obtained in gastric cancer cells treated with purified cimiside E purified from *Cimicifuga heracleifolia* (Guo et al., 2009).

A similar study was performed in ER-positive MCF-7 and ER-negative MDA-MB-435 cells, to examine the effect of CRE constituents on growth

inhibition cell cycle arrest (Einbond et al., 2004). The authors found that the triterpene glycosides actein, 23-*epi*-26-deoxyactein, and cimiracemoside A inhibited cell growth in MCF-7 cells and induced cell cycle arrest in the G1 phase. The most potent compound, actein, decreased the levels of cyclin D1, cdk4, and other factors, thereby contributing to arrest in the G1 phase (Einbond et al., 2004).

Fang et al. showed that several cycloartane triterpenoids (namely, 25-*O*-acetylcimigenol-3-*O*-β-D-xylopyranoside, 25-chlorodeoxycimigenol-3-*O*-β-D-xylopyranoside, 25-*O*-acetylcimigenol-3-*O*-α-l-arabino-pyranoside, and 23-*O*-acetylcimigenol-3-*O*-β-D-xylopyranoside) inhibited the growth of both MCF-7 cells and drug-resistant R-MCF-7 cells by inducing apoptosis *via* p53-dependent mitochondrial pathways (Fang et al., 2011). Recently, experiments in early liver cancer in mice showed that actein ameliorated diethylnitrosamine-induced steatohepatitis, fibrosis and inflammation (which may progress to cancer) and reduced proliferation markers such as cyclin Ds or p53 as well as stem cell markers (*e.g.* CD133) and up-regulation of hypoxia-inducible factor 1-α (Hif-1α) and VEGFR1 and may prevent cancer progression (Xi and Wang, 2017).

Further insights into the pharmacological mechanisms underlying the effects of CREs were obtained by performing microarray analyses of liver samples taken from Sprague-Dawley rats treated with a CRE enriched with triterpene glycosides (Einbond et al., 2012). The authors found that CRE markedly downregulated mitochondrial oxidative phosphorylation genes; in contrast, genes involved in phospholipid biosynthesis and remodeling, PI3-kinase, and sphingosine signaling were upregulated, driven largely by an upregulation of several isoforms of phospholipase C. Additional experiments revealed that CRE further repressed the expression of cyclin D1 and the cell growth regulator ID3 (inhibitor of DNA binding 3) in hepatic HepG2 cancer cells, thus inhibiting their proliferation (Einbond et al., 2012). *In vivo*, however, the proliferation rate of 7,12- dimethylbenz[a]anthracene induced mammary breast cancer tumors in OVX rats was not increased by treatment with various doses of CRE for 6 weeks in clear contrast to mestranol treatment. showing No significant difference in tumor number or size between CRE-treated rats and vehicle-treated controls was observed (Freudenstein et al., 2002) .

Finally, the question of whether CRE has estrogenic effects was addressed in a 6-month clinical study in peri- and postmenopausal women who received two different doses of a unique CRE preparation (Liske et al., 2002). Besides the effect on climacteric symptoms, CRE had no effect on vaginal cytology, indicating a lack of estrogenic effect on this organ. Likewise, the lack of significant changes in the levels of gynecologically relevant hormones did not indicate an overall estrogenic effect (Liske et al., 2002). Building on this evidence, in a subsequent clinical study in 136 breast cancer survivors after conventional therapy (surgery, radiation and adjuvant chemotherapy) were randomly assigned to receive either tamoxifen alone (*i.e.*, usual care) or tamoxifen plus the CRE BNO 1055 for the treatment of hot flushes (Hernández Munoz and Pluchino, 2003). The authors found that nearly half of the patients in the intervention group (tamoxifen plus CRE) experienced no hot flushes; moreover, the overall number and severity of hot flushes were significantly lower in the intervention group compared to the usual care group ($p < 0.01$) (Hernández Munoz and Pluchino, 2003). In addition, Henneicke-von Zepelin et al. performed a retrospective pharmacoepidemiologic study of 18,861 breast cancer survivors, 1102 of whom had received CRE treatment; their analysis revealed that although CRE treatment was not associated with an increased risk of recurrence, it was associated with prolonged disease-free survival (Henneicke-von Zepelin et al., 2007). (Fig. 1)

Thus, a growing body evidence suggests that CREs do not exert estrogenic effects, do not promote tumor growth, and do not induce proliferative activity *in vitro*. On the other hand, preliminary clinical data suggest that CREs may be safely given to breast cancer survivors (Henneicke-von Zepelin et al., 2007; Hernández Munoz and Pluchino, 2003; Rostock et al., 2011; Ruan et al., 2019). However, additional clinical studies are clearly warranted before a definite statement could be given.

AMPK and cell proliferation/cancer

Many of CRE's documented effects both in experimental cancer models and in patients may be explained by activation of the AMPK pathway.

Hardie reviewed the evidence indicating that AMPK is the central cellular energy sensor, playing a key role in metabolic disorders and cancer (Hardie, 2011). Indeed, one of the pathway's upstream targets, the tumor suppressor LKB1 (liver kinase B1), phosphorylates—and thus activates—AMPK. Activated AMPK then inhibits cellular growth and proliferation by activating catabolic metabolic processes and inhibiting anabolic metabolic processes. For example, AMPK inhibits mTOR (mechanistic target of rapamycin) complex 1, a nutrient-sensitive master regulator of cell growth, angiogenesis, and metabolism that is activated by growth factors, particularly in tumors (Gwinn et al., 2008; Shackelford and Shaw, 2009).

Several *in vitro* and/or *in vivo* assays revealed that metformin, the indirect activator of AMPK, induces cell cycle arrest in a wide range of tumor models, including breast cancer (Zhuang and Miskimins, 2008), nasopharyngeal carcinoma (Zhao et al., 2011), renal cell carcinoma (Liu et al., 2013), esophageal squamous cell carcinoma (Cai et al., 2015), and prostate cancer (Ben Sahra et al., 2008).

Hence AMPK serves as the link between the therapeutic effects of metformin on metabolic syndrome and cancer, making AMPK a promising target for cancer prevention and therapy (Luo et al., 2010). Indeed, many clinical studies using metformin in cancer indications have been performed to date; a search pf PubMed conducted on August 23, 2020, using the search term ["metformin" AND "cancer" AND "clinical"] yielded 2220 publications. We therefore refer the reader to several relevant reviews (Ben Sahra et al., 2010; Gallagher and LeRoith, 2011; Li, 2011; Luo et al., 2010; Rizos and Elisaf, 2013). In addition, ClinicalTrials.gov currently lists 34 previous or ongoing clinical trials to assess the effect of metformin on outcome in various types of cancer (Choi, 2011, 2013a, b; Cui et al., 2013; Lee and Choi, 2014; Li et al., 2007; Nisslein and Freudenstein, 2003; Qiu et al., 2007; Seidlova-Wuttke et al., 2012; Wuttke et al., 2003b).

Summary and future outlook

A large body of evidence indicates that CREs are effective in the treatment of climacteric symptoms (Aly, 2009; Bai et al., 2007; Drewe et al., 2013; Friederichsen et al., 2020; Liske et al., 2002; Lopatka et al., 2007; Nappi et al., 2005; Osmers et al., 2005; Ross, 2012; Schellenberg et al., 2012; Wuttke et al., 2006, 2003b). In addition, many of the postmenopausal symptoms attributed to estrogen deficiency may be improved by activating the AMPK pathway, and CREs have effects that are similar to the effects of direct and/or indirect activators of AMPK. Moreover, several constituents isolated from CREs such as 23-*epi*-26--deoxyactein, have been shown to activate AMPK both *in vitro* and *in vivo*.

It is important to note that this review does not question the current view regarding the mode of action of CREs, which is based on modulation of the serotonergic system by CREs; rather, we have attempted to extend this view. Menopause is no longer regarded simply as an isolated condition, but a manifestation of a general aging process that is not restricted to the reproductive organs. Indeed, menopause can be viewed as a systemic condition with manifestations in a wide range of organ systems. The elements that connect these changes include metabolic dysregulation and age-related cellular dysfunction caused a variety of factors such as an accumulation of oxidative stress. In this respect, AMPK plays a central role, thus serving as a master switch integrating nutritional resources, cellular energy status and demand, and the extent of ROS production (Hardie et al., 1998).

Identifying and recognizing AMPK as an important target of *Cimicifuga racemosa* extracts opens new insights into the development of other therapeutic indications beyond the treatment of climacteric symptoms

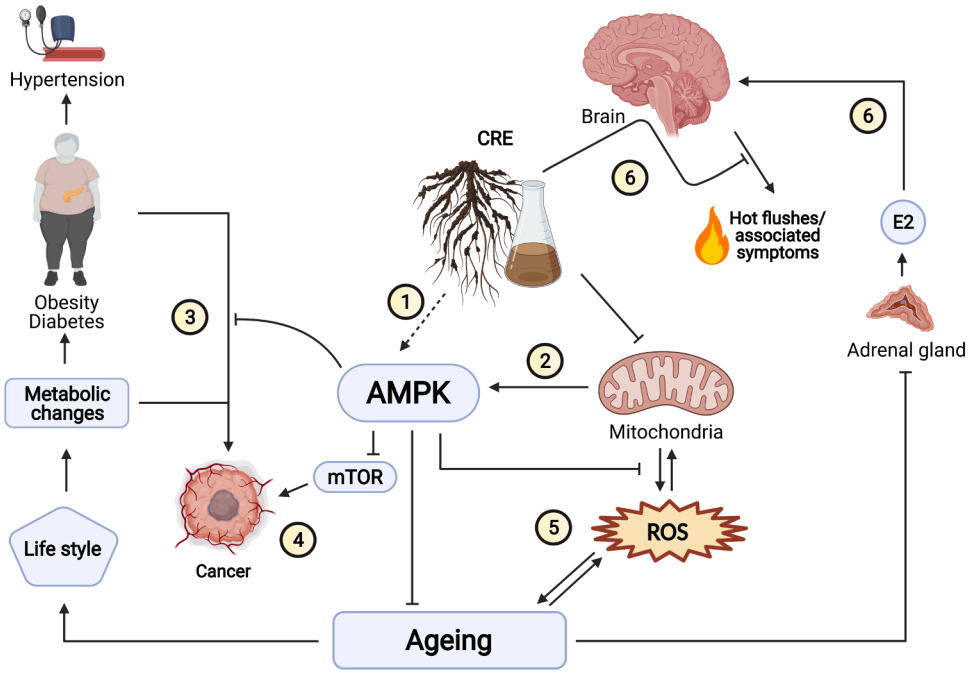

Fig. 1. Action of *Cimicifuga racemosa* on AMPK-dependent metabolic pathways and hot flushes. AMPK is directly activated by CRE (1) or indirectly by inhibiting mitochondrial respiration (2). AMPK activation regulates insulin sensitivity and energy metabolism (3), thereby reducing the risk of obesity, diabetes, and associated other conditions such as hypertension and cardiovascular diseases. Furthermore, AMPK can attenuate malignant cell proliferation by inhibiting mTOR (4), which together with the metabolic effects may prevent the proliferation of cancer cells. In addition, CRE's direct effects on mitochondrial integrity and function may contribute to metabolic regulation, reducing ROS formation (5) and therefore mitigating aging-related processes. In the brain, these metabolic mechanisms are linked to estradiol signaling and are therefore related to the regulation of body temperature and vascular effects (6), thus underlying hot flushes and other symptoms typically associated with the early stages of menopause. Overall, these new mechanisms of action in which CREs affect AMPK activation and mitochondrial regulation greatly extend our understanding of menopausal complaints in the context of age-related oxidative stress and metabolic dysregulation, revealing new therapeutic implications. AMPK, AMP-activated protein kinase; CRE, *Cimicifuga racemosa* extract; E2, estradiol; mTOR, mechanistic target of rapamycin; ROS, reactive oxygen species.

in peri- and postmenopausal women in the sense of repurposing. Starting from age-related menopausal symptoms, the aging process itself and its clinical manifestations now come into focus. However, before definite recommendation can be made, clinical studies are needed to confirm this view.

Declaration of Competing Interests/Conflict of Interest

JD and GB are employees at Max Zeller Soehne AG in Romanshorn, Switzerland. CC declares no conflict of interest.

CRediT author statement

All authors designed the structure and organization of the review, and outlined the major messages. JD and CC performed literature search, wrote the paper draft, designed the figures, tables and the graphical abstract. GB performed literature search, corrected and finalized the draft.

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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