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Urocortin 3 in obesity and type 2 diabetes

Sina Antony Kavalakatt

Doctoral Programme in Biomedicine,

Faculty of Medicine,

University of Helsinki

And

Dasman Diabetes Institute,

Kuwait

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Supervised by:

Professor Jaakko Tuomilehto
Department of Public Health,
University of Helsinki, Helsinki, Finland
and
National Institute of Health and Welfare (THL),
Helsinki, Finland

Adjunct Prof. (Docent) Heikki A. Koistinen
Department of Medicine, University of Helsinki
and
Helsinki University Hospital, Helsinki, Finland.
Minerva Foundation Institute for Medical Research,
Helsinki, Finland

Professor Fahd Al-Mulla
Molecular Pathology and Genomic Medicine, Kuwait University
and
Dasman Diabetes Institute, Kuwait

Reviewed by:

Professor Leo Niskanen
Department of Internal Medicine, Päijät-Häme Joint Authority for Health and Wellbeing;
and University of Eastern Finland, Finland

Professor Kirsi Virtanen
University of Eastern Finland, Finland

Opponent:

Professor Anna Krook
Department of Physiology and Pharmacology,
Integrative Physiology,
Karolinska Institutet,
Stockholm, Sweden

To the pursuit of knowledge

To my dearest family

Abstract

Diabesity is a major health burden worldwide, particularly in Kuwait, where several epidemiologic studies reveal the parallel escalation of the prevalence of obesity and diabetes. There is still a lack of complete understanding of the pathways and their interactions triggering the development of obesity-related co-morbidities. Urocortin 3 (UCN3) is a novel neuropeptide implicated in the regulation of food intake, energy homeostasis, cardioprotection, and identified as a regulator of insulin secretion and as a marker for functional pancreatic β-cells. Hence, UCN3 could be a potential therapeutic target for managing metabolic diseases.

In this thesis we have assessed UCN3 expression in two cross-sectional populations living in Kuwait: adults with obesity and type 2 diabetes (T2D) and children with overweight and obesity. In addition, we have examined associations of UCN3 with metabolic markers and stressors and assessed the effect of UCN3 overexpression on signaling pathways of insulin, glucose uptake, endoplasmic reticulum (ER) stress, and heat shock response in an adipocyte cell model.

In study (I), we investigated the circulating UCN3 levels in the plasma of adults with obesity and T2D. The effect of inflammatory microenvironment in adipose tissue on the expression of UCN3 and the effect of physical exercise training on UCN3 expression was also investigated. We demonstrated that UCN3 expression levels were impaired in response to an inflammatory microenvironment, obesity, and T2D.

In study (II), the effect of UCN3 overexpression on apoptotic, ER, and heat-shock stress response pathways was studied in 3T3L1 adipocytes. We showed that the overexpression of UCN3 attenuated markers of apoptosis, inflammation, ER stress, and heat shock response. These events were associated with improved glucose uptake and insulin signaling.

We showed that the levels of UCN3 and the other corticotropin-releasing factor (CRF) family are impaired with obesity both in plasma and peripheral blood mononuclear cells obtained from children. In studies (III) and (IV), the circulating levels of UCN3 were assessed in children with different levels of adiposity. Metabolic stressors such as palmitic acid and high glucose concentrations differentially

modulated the neuropeptide levels in human monocytic cell line (THP1) cells depending on the duration of the exposure.

The homeostasis of energy balance and metabolism is regulated by the central nervous system and peripheral mechanisms. The progression of obesity and T2D is associated with disturbances in this homeostasis, dysregulation of insulin secretion, and alterations in inflammatory and stress response pathways. Most of the thesis findings were shown for the first time and highlighted the role of UCN3 in metabolic dysregulations in obesity and T2D. UCN3 might be a promising marker in future approaches to monitor the progression of obesity, T2D, and related metabolic co-morbidities.

TIIVISTELMÄ

Diabetes on merkittävä terveystaakka maailmanlaajuisesti ja erityisesti Kuwaitissa, jossa useat epidemiologiset tutkimukset ovat todenneet lihavuuden ja diabeteksen esiintyvyyden lisääntyvän samanaikaisesti. Tieto lihavuuteen liittyvien sairauksien syntyyn vaikuttavista eri reiteistä sekä niiden vuorovaikutuksista on edelleen puutteellista. Urokortiini 3 (UCN3) on neuropeptidi, joka säätelee ruokailua ja energiatasapainoa sekä suojaa sydäntä. UCN3 säätelee insuliinieritystä ja se on tunnistettu haiman kypsien β-solujen merkkiaineeksi. UCN3 voi olla mahdollinen terapeuttinen kohde hoidettaessa aineenvaihduntasairauksia.

Tässä väitöskirjatyössä tutkittin UCN3:n ilmentymistä kahdessa Kuwaitissa asuvassa väestössä: lihavilla ja tyypin 2 diabetesta (T2D) sairastavilla aikuisilla sekä ylipainoisilla ja lihavilla lapsilla. Lisäksi selvitettiin yhteyksiä UCN3:n ja aineenvaihdunnan merkkiaineiden sekä stressitekijöiden välillä. UCN3:n yli-ilmentymisen vaikutusta insuliinin signaalinsiirtoon, glukoosin soluunottoon, endoplasmisen kalvoston stressiin sekä lämpösokkivasteeseen tutkittiin rasvasolumallissa.

Tutkimuksessa I tutkimme plasman UCN3 pitoisuuksia lihavilla ja T2D:ta sairastavilla aikuisilla. Tutkimme myös tulehduksellisen mikroympäristön vaikutusta UCN3:n ilmentymiseen sekä liikuntaharjoittelun vaikutusta UCN3:n ilmentymiseen. Osoitimme, että UCN3:n ilmentyminen vähenee vasteena tulehdukselliselle mikroympäristölle, lihavuudelle ja T2D:lle. Liikuntaharjoittelu vaikutti rasvakudoksen UCN3:n ilmentymiseen.

Tutkimuksessa II tutkittiin UCN3:n yli-ilmentymisen vaikutusta apoptoosin, endoplasmisen kalvoston stressin sekä lämpösokkivasteen signaalinsiirtoreitteihin 3T3L1-rasvasoluissa. UCN3:n yli-ilmentyminen vähensi apoptoosia, tulehdusta, endoplasmisen kalvoston stressiä sekä lämpösokkivasteen merkkiaineita. Nämä tapahtumat liittyivät parantuneeseen glukoosin soluunottoon ja insuliinin signaalinsiirtoon.

Tutkimuksissa III ja IV plasman UCN3 pitoisuuksia arvioitiin ylipainoisilla ja lihavilla lapsilla. Lasten ylipaino ja lihavuus vaikuttivat UCN3:n ja kortikotropiinia vapauttavan tekijän (CRF) ilmentymiseen plasmassa ja perifeerisen veren

mononukleaarisoluissa. Metaboliset stressitekijät, kuten palmitaatti ja korkea glukoosipitoisuus, vaikuttivat myös näiden neuropeptidien ilmentymiseen THP1-soluissa altistuksen kestosta riippuvalla tavalla.

Sekä keskushermosto että perifeeriset mekanismit säätelevät energiatasapainon ja aineenvaihdunnan homeostaasia. Lihavuuden ja T2D:n eteneminen näyttää liittyvän häiriöihin tässä homeostaasissa, muutoksiin insuliinin erityksessä, sekä poikkeamiin tulehdus- ja stressivasteissa. Väitöskirjatutkimuksessani on useita alkuperäishavaintoja ja se tuo uutta tietoa UCN3:sta lihavuudessa ja T2D:ssa. UCN3 saattaa olla uusi lupaava merkkiaine lihavuuden, T2D:n ja näihin liittyvien sairauksien etenemisen seuraamiseksi.

Contents

List of original publications

This thesis is based on the following publications:

I. **Kavalakatt, S**., Khadir, A., Madhu, D., Hammad, M., Devarajan, S., Abubaker, J., Al-Mulla, F., Tuomilehto, J., & Tiss, A. (2019). Urocortin 3 Levels Are Impaired in Overweight Humans with and Without Type 2 Diabetes and Modulated by Exercise. Frontiers in Endocrinology, 10, 762. Doi.org/10.3389/fendo.2019.00762

II. **Kavalakatt, S**., Khadir, A., Madhu, D., Devarajan, S., Warsame, S., AlKandari, H., AlMahdi, M., Koistinen, H. A., Al-Mulla, F., Tuomilehto, J., Abubaker, J., & Tiss, A. (2022). Circulating levels of urocortin neuropeptides are impaired in children with overweight. Obesity (Silver Spring, Md.), 30(2), 472–481. https://doi.org/10.1002/oby.23356

III. **Kavalakatt, S**., Khadir, A., Kochumon S., Madhu, D, Devarajan, S., Hammad, M., Alamaldin N., Ahmed R., Warsame S., AlKandari H., AlMahdi M., Koistinen H.A., Al-Mulla F., Tuomilehto J., Abubaker J., and Tiss A. (2022), Levels of Urocortin neuropeptides are impaired in the PBMCs of overweight children. Nutrients ;14(3):429. https://doi.org/10.3390/nu14030429

IV. **Kavalakatt, S**., Khadir, A., Madhu, D., Koistinen, H. A., Al-Mulla, F., Tuomilehto, J., Abubaker, J., & Tiss, A. (2021). Urocortin 3 overexpression reduces ER stress and heat shock response in 3T3-L1 adipocytes. Scientific Reports, 11(1), 15666. Doi.org/10.1038/s41598-021-95175-4

The publications are referred to in the text by their roman numerals.

Abbreviations

ACC	Acetyl-CoA carboxylase
AHS	Adult human study cohort
ALT	Alanine transaminase
AMPK	Adenosine monophosphate activated protein kinase
AST	Aspartate transaminase
ATF6	Activating transcription factor 6
CaMKII	Calcium/calmodulin dependent kinase II
CCL2	chemokine (C-C motif) ligand 2
Ccr	Creatinine clearance test
CHOP	CCAAT/enhancer-binding protein(C/EBP) homologous protein
COP	Childhood obesity program
C-peptide	Connecting peptide
CPET	Cardiopulmonary exercise test
CREB	Ca^{2+}/ cAMP response element binding protein
CRF/CRH	Corticotropin releasing factor/ Corticotropin releasing hormone
CRFR	Corticotropin releasing factor receptor
dKO	Double knockout
ECM	Extracellular matrix
FC	Fold change
FPG	Fasting plasma glucose
GPCRs	G-protein coupled receptors
GRP	G regulated protein 78
HF	Heart failure
HFD	High fat diet
HG	High glucose
HPA	Hypothalamus- pituitary-adrenal axis
hsCRP	High sensitivity-C-reactive protein
HSP	Heat shock protein
I/R	Ischemia-reperfusion

ICV	Intracerebroventricular injection
JNK	c-Jun N-terminal kinase
Ki	Inhibition constant
LIPE	Lipase E
LPC	Lipophosphatidyl choline
LPL	Lipoprotein Lipase
MaCM	Macrophage conditioned medium
MAPK	Mitogen activated protein kinase
max HR	Maximum heart rate
Mt1	Metallothionine 1
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
OE	Overexpression
OR	Odds ratio
PA	Palmitic acid
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PVN	Paraventricular nucleus
SAT	Subcutaneous adipose tissue
SBP	Systolic blood pressure
T2D	Type 2 diabetes
T4	Thyroxine
TGL	Triglycerides
TRH	Thyrotropin releasing hormone
TSH	Thyroid-stimulating hormone
tz/tz	tau-lacZ reporter gene
UCN	Urocortin
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximum oxygen consumption
WT	Wild type
ZAG	Zinc alpha 2-glycoprotein

1 Introduction

In the modern lifestyle, high exposure to stress along with overnutrition and sedentary lifestyle are correlated with many health problems, including both psychological (mood disorders) and pathophysiological (obesity, diabetes, metabolic syndrome, and cardiovascular diseases) (Charmandari, Tsigos, and Chrousos 2004; Kyrou and Tsigos 2007). Multiple studies have highlighted the association of obesity with increased risk of metabolic diseases, including insulin resistance, type 2 diabetes (T2D), dyslipidemia, and non-alcoholic fatty liver disease (NAFLD) (Ghemrawi, Battaglia-Hsu, and Arnold 2018). Obesity results from excessive adipose tissue accumulation due to an imbalance between energy intake and expenditure. Exposure to elevated concentrations of glucose and lipids in blood circulation leads to glucotoxicity and lipotoxicity, inducing stress signaling such as endoplasmic reticulum (ER) stress and oxidative stress (Ghemrawi, Battaglia-Hsu, and Arnold 2018). Complex physiological systems maintain a dynamic control in our body to sustain homeostasis, and the increase in stress response is any actual or perceived threat to this homeostasis. Under such conditions, several adaptive responses affecting central nervous and peripheral systems in the body are activated.

Adipose tissue is now recognized as an endocrine organ producing several adipokines triggering low-grade inflammation and interacting with multiple processes in different organs (Kershaw and Flier 2004a). It responds to the various hormonal and central nervous systems and secretes factors such as leptin, adiponectin, resistin, and plasminogen activator inhibitor-1 (Kershaw and Flier 2004b). Excessive fat accumulation promotes the free fatty acids, and adipokines release into blood circulation, modulating insulin sensitivity inflammatory responses and altering glucose and lipid homeostasis.

The two main arms that constitute the stress response system are; the neuroendocrine system – the hypothalamus-pituitary-adrenal (HPA) axis – and the autonomous nervous system with central and peripheral components (Kyrou and Tsigos 2007). The HPA axis releases and orchestrates various substances that coordinate physiological responses. The stress response can rapidly activate both the HPA axis and autonomous nervous system, thereby regulating glucocorticoid

synthesis and release from the cortex of the adrenal gland. The complex interaction between the components of this system is relevant in dysfunctional stress circuits that result in so-called neuro-psycho-pathologies (Joëls and Baram 2009).

The modulated adaptive stress responses have further consequences on physiologic processes such as growth, metabolism, inflammation, blood circulation, immune response, and neurodegenerative disorders (Habib, Gold, and Chrousos 2001). Positive reaction enables restoration of homeostasis; however, when this is prolonged, the response may develop into adverse pathological outcomes leading to the development and progression of obesity, diabetes, sleep disorders, cardiovascular diseases, and metabolic syndrome (Dallman et al. 2003; Kyrou and Tsigos 2007).

The important question is how to identify and monitor the progression of these processes from positive stress to maladaptive stress considering that the threshold for this transition may depend upon individual genetic predisposition (Sharma et al. 2016).

Wylie Vale discovered a corticotropin-releasing factor or corticotropin-releasing hormone (CRF/ CRH) as the central mediator of the neuroendocrine and sympathetic stress response (Vale et al. 1981). CRF stimulates the release of adrenocorticotropic hormone (ACTH) and subsequently glucocorticoids which mediate numerous physiological and metabolic reactions (Saffran and Schally 1955; Saffran, Schally, and Benfey 1955). These reactions translate into the activation of the cardiovascular system, anti-inflammatory effects, energy mobilization, and diminished digestive functions, among others. The CRF-system comprised of; CRF, Urocortins (UCN) − UCN1, UCN2, UCN3 − and the receptors corticotropin-releasing factor receptors (CRFR)1 and CRFR2, plays a role in the regulation of energy homeostasis, cardiovascular function, food intake, appetite, and behavior (Hashimoto et al. 2004; Fekete et al. 2007; Spina et al. 1996). UCN3 is the most recently discovered neuropeptide implicated in regulating insulin secretion, glucose uptake, and energy homeostasis, among other physiological roles. However, the role of UCN3 in obesity and related metabolic disorders is still unclear.

This thesis comprises four studies that examine the role of UCN3 in obesity and T2D. These studies report for the first time 1) the dysregulation of UCN3 in obesity

in adults and children, 2) impaired plasma and adipose tissue UCN3 levels in people with T2D, 3) modulation of UCN3 by exercise in adipose tissue from adults, 4) decreased UCN3 transcript levels with obesity in peripheral mononuclear blood cells (PBMCs) in children, 5) UCN3 overexpression reduces ER stress and heat shock response and improves insulin signaling and glucose uptake in 3T3L1 adipocytes, and 6) metabolic stressors such as palmitic acid and high glucose concentrations decreased UCN3 levels in human monocytic cell line (THP1) cells concomitantly with exacerbated ER stress and inflammation.

2 Review of literature

2.1 Obesity, diabetes, and adipose tissue

The global prevalence of obesity and associated metabolic complications have increased substantially in recent years, becoming a major epidemiological challenge on healthcare resources. The number of overweight and obese adults has risen dramatically in the last decades (Hurt et al. 2010). The incidence of childhood obesity is also increasing at alarming rates. Kuwait is one of the top 3 countries with the highest prevalence of diabetes (24.9 %) (Sun et al. 2021). The prevalence of childhood and adolescent obesity in the country is also alarmingly high (Farrag, Cheskin, and Farag 2017).

Obesity is the excessive amount of body fat. In adults, obesity is often assessed using body mass index (BMI) calculated as the bodyweight divided by the square of body height (kg/m^2). However, considering only BMI is misleading under certain settings due to differences in genetics, fat distribution, and percentage of body adiposity among different countries (Goh et al. 2004). Individuals with a BMI of 30 kg/m^2 or greater are classified as obese ('Obesity: preventing and managing the global epidemic. Report of a WHO consultation' 2000). Obesity in children is defined based on percentile growth charts from the Centre for Disease Control and Prevention. In children and adolescents aged 19 years or younger, obesity is defined as at or above 95^{th} percentile, whereas overweight is between 85^{th} and 95^{th} percentile (Kuczmarski et al. 2002).

Of obese children, 55 % become obese adolescents, and 80% of obese adolescents are likely to become obese adults. Obese individuals are at a higher risk of developing insulin resistance, dyslipidemia, T2D, heart disease, NAFLD, and other metabolic disorders and have an elevated risk for adverse psychological problems (Schiller et al. 2012). Hence, obese children are at a higher risk of developing a range of endocrine and other metabolic disorders. Unsurprisingly, obese children also have many cardiovascular risk factors observed in obese adults, such as hypertension, dyslipidemia, T2D, and metabolic syndrome (Simmonds et al. 2016).

Several studies have highlighted the highly integrated role of inflammation and stress responses that explains the difficulty in finding effective therapies to manage obesity and related complications. Complex mechanisms including chronic low-grade inflammation and metabolic stress response are activated in obesity (Figure 1). Metabolic stress has been shown to alter insulin responses by modulating several signaling cascades in adipose tissue, skeletal muscle, and liver (Rask-Madsen and Kahn 2012). Of these, the mitogen-activated protein kinase (MAPK) signaling, heat shock response (HSR), and ER response (Khadir, Kavalakatt, et al. 2018b; Baturcam et al. 2014; Khadir et al. 2015) are the main stress response pathways. Metabolic stress also activates the HPA axis supporting the complex association between stress, obesity, metabolic syndrome, and altered HPA axis activity (Bose, Oliván, and Laferrère 2009).

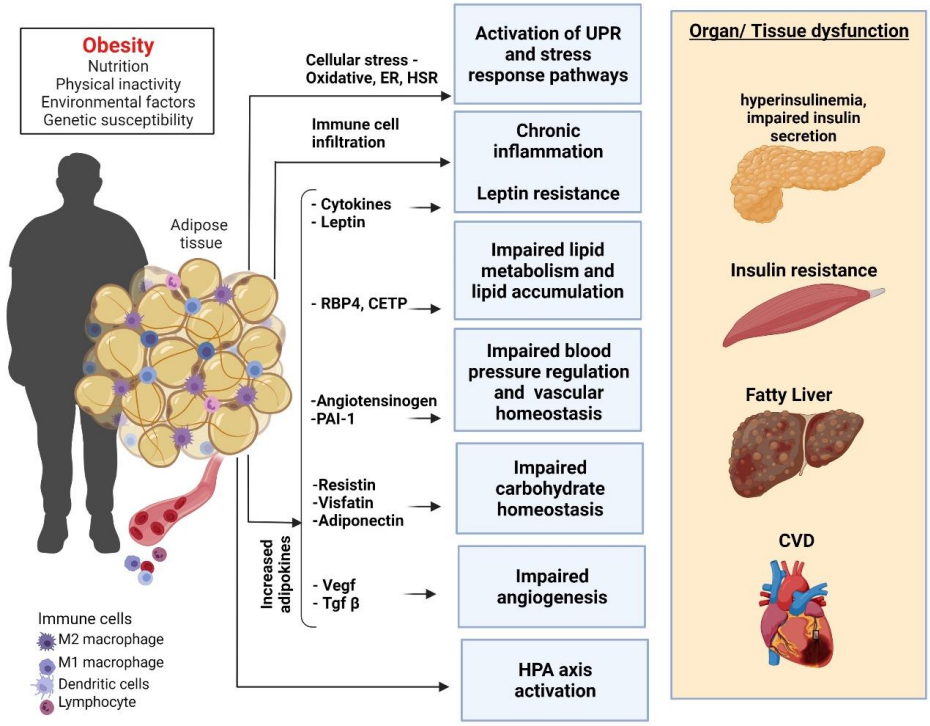

Figure 1: Physiological and metabolic changes in obesity regulated by adipose tissue resulting in metabolic diseases. *Obesity results from multiple factors, including excessive nutrition, physical inactivity, environmental factors, and genetic susceptibility. The hypertrophy of adipose tissue can activate several complex pathways via increased cellular stress, immune cell infiltration, and increased*

secretion of adipokines. Meanwhile, in an obese state, expansion of β-cell mass is associated with increased insulin production and further exacerbated by leptin resistance resulting in hyperinsulinemia and insulin resistance in insulin-responsive tissues. Obese individuals are at a higher risk of developing NAFLD, dementia, and cardiovascular disease. Obesity is also associated with increased production of cortisol, the main glucocorticoid, and hyperactivation of the HPA axis, causing mood disorders and dysregulation of stress responses, food intake, and fat mass. UPR- unfolded protein response, HSR- heat shock response, ER- endoplasmic reticulum, RBP4- Retinol Binding Protein 4, CETP-Cholesteryl Ester Transfer Protein, PAI-1 Plasminogen activator inhibitor-1, VEGF Vascular endothelial growth factor A, TGFβ Transforming growth factor-beta 1. Created with Biorender.com

The MAPKs consist of the extracellular signal-regulated kinase (ERK), c-Jun NH_2 terminal kinase (JNK), and p38 MAPK. They are activated in response to physiological and pathophysiological stimuli and are involved in various biological processes, including metabolism and response to stress and inflammation. These stress or growth factor induced-kinases act in a context, duration, and magnitude-dependent manner; therefore, tight control of their responses is crucial for homeostasis (Kim and Choi 2010). The HSR involves activating heat shock proteins (HSP), mainly HSP110, HSP90, HSP70, HSP60, HSP40, and other small HSPs. Recent studies have shown that HSR is impaired in obesity-induced insulin resistance. Moreover, therapies modulating certain HSPs improve glucose homeostasis insulin sensitivity and suppress chronic inflammation (Kondo et al. 2011). Impaired ER stress response occurs in obesity and diabetes (Hotamisligil 2010). Normal ER function is not restored when the unfolded protein response (UPR) system fails to lead to chronic ER stress. The three arms of the UPR consist of activating transcription factor-6 (ATF6), inositol requiring enzyme-1α (IRE1α), and protein kinase RNA such as endoplasmic reticulum kinase (PERK) act as ER stress sensors. The master regulator of UPR, glucose-regulated protein 78 (GRP78) chaperone, is bound to these sensors under normal physiologic conditions. However, metabolic insults such as excessive fat storage, reactive oxygen species accumulation, and disturbed calcium homeostasis dissociate them from GRP78 and thus leading to their activation (Wang and Kaufman 2012).

Adipose tissue is a major endocrine organ that stores excess energy as triglycerides. Multiple adipokines, including cytokines, hormones, and chemokines such as TNFα, CCL2/MCP1, IL6, leptin, resistin, visfatin, PAI1, RBP4, and adiponectin are secreted from adipose tissue (Jung and Choi 2014). These adipokines are involved in energy homeostasis. When energy is required between meals or during physical activity, lipolysis of triglycerides releases fatty acid into circulation, which can be used as an energy source. Adipose tissue is an insulin-responsive tissue. Insulin enhances the differentiation of pre-adipocytes into adipocytes, stimulates lipogenesis, increases the uptake of glucose and fatty acids, and reduces lipolysis.

Triglycerides are stored either by an increase in the number of adipocytes (hyperplasia) or the size of adipocytes (hypertrophy) (Hausman et al. 2001). Adipose tissue consists of multiple cell types, including pre-adipocytes, adipocytes, immune cells, and endothelial cells. It was initially shown that the number of adipocytes is determined in childhood and adolescence and remains constant in adulthood. Hence, fat mass increase in adults occurs mainly through hypertrophy (Spalding et al. 2008). Recent advances in technology have allowed accurate determination of fat cell turnover, showing that weight gain is facilitated by increasing adipocyte size and mass (Arner and Rydén). Adipose tissue hypertrophy has been suggested to play a critical role in developing insulin resistance and metabolic diseases (Weyer et al. 2000). Free fatty acids, products of triglyceride lipolysis, serve as the stimulus of cytokine production, leading to low-grade adipose tissue inflammation; this contributes to obesity-associated peripheral insulin resistance and metabolic complications (Boden 1997). The combinations of insulin resistance, metabolic syndrome, and adipose tissue accumulation are major predictors of T2D (Antonio-Villa et al. 2020).

The metabolic role of different adipose tissue is of growing clinical interest. White adipose tissue (WAT) is an endocrine organ with a primary role in energy storage. Brown adipose tissue (BAT) plays a role in thermogenesis by converting chemical energy to heat. WAT and BAT functions ensure balanced energy homeostasis. WAT is a highly active organ that secretes several hormones that modulate inflammation, glucose metabolism, lipid metabolism, insulin resistance, and coagulation. These mediators lead to the development and progression of metabolic diseases. WAT consists of several cell types and can be classified into visceral, subcutaneous, and

pericardial adipose tissue (Merkel, Schmid, and Iwen 2019). Adipose tissue beneath the skin is called subcutaneous adipose tissue (SAT), and the one lining the internal organs is called visceral adipose tissue (VAT) (Mittal 2019).

The development of metabolic disorders is associated with insulin resistance of SAT aggravated by morbid obesity, aging, nutrition, low physical activity, and genetic factors (Pandžić Jakšić and Grizelj 2016). Morbidly obese individuals have higher pro-inflammatory gene expression in SAT than VAT, indicating that SAT contributes to obesity-related inflammation (Spoto et al. 2014). The best option of reducing SAT and VAT is lifestyle intervention, including nutrition and physical activity, apart from drug treatments and surgery.

Physical activity is a part of a healthy lifestyle and can be non-pharmacological therapy for non-communicable diseases, including obesity, insulin resistance, diabetes, and cardiovascular diseases (O'Leary et al. 2006). The molecular mechanisms for the beneficial effects of exercise are complex and not yet fully elucidated; however, several studies have reported the protective benefits against HFD-induced inflammation are mediated through the decreased production of inflammatory mediators and reduced infiltration of inflammatory cells into the adipose tissue (Kawanishi et al. 2010). Other studies have also shown the improvement of leptin and insulin signaling by exercise in the brain, liver, and skeletal muscle (Park et al. 2005; Akimoto et al. 2005; Király et al. 2010). Reduction of BMI in response to bariatric surgery mitigated ER stress in insulin-resistant obese individuals; hence, a decrease in BMI through physical exercise could also have similar effects on ER stress (Gregor et al. 2009). Physical exercise also modulates HSPs in individuals with obesity and T2D (Khadir, Kavalakatt, et al. 2018a; Abubaker et al. 2013).

2.2 Discovery of CRF family neuropeptides and their tissue distribution

Two independent research groups identified the presence of a factor responsible for the secretion of adrenocorticotropic hormone (ACTH), and this was identified and named as corticotropin-releasing factor (CRF) (Saffran and Schally 1955; Saffran, Schally, and Benfey 1955). CRF was the first factor detected from the hypothalamus and was suggested to have other regulatory functions (Fink 1981). CRF-related

peptide, Urocortin 1 (UCN1) was discovered due to its homology to CRF in 1995 (Fink 1981). UCN1 was also shown to induce ACTH secretion and bind to type 1 and type 2 CRF receptors (CRFR1 and CRFR2). Two other members were identified and initially named as Stresscopin and Stresscopin-related peptides (Lewis et al. 2001; Reyes et al. 2001). These peptides are now named UCN3 and UCN2, respectively. The CRF family consists of four main peptides, CRF, UCN1, UCN2, and UCN3, along with their receptors CRFR1 and CRFR2. All these peptides in the CRF family are secreted as pro-peptides with a signal peptide that is proteolytically cleaved to form the bioactive neuropeptides (Hook et al. 2008).

These neuropeptides and their receptors are present in the brain and peripheral tissues (Figure 2), where they show differential ligand-receptor interaction (Deussing and Chen 2018). CRF is strongly expressed in the hypothalamus, and it regulates the activity of the HPA axis (Sawchenko and Swanson 1985). Furthermore, CRF is expressed in the gastrointestinal (GI) tract (Buckinx et al. 2011), skin, the immune system, including spleen, mast cells, thymus, endothelial cells, and macrophages (Karalis et al. 1991; Baigent 2001), as well as in reproductive systems (Kalantaridou et al. 2007). UCN1 has been reported to have much-restricted distribution in the brain. UCN1 is expressed in the gastrointestinal (GI) tract, thymus, spleen, cardiac myocytes, and immune system (Cureton et al. 2009; Borges et al. 2015). UCN2 is expressed in the hypothalamus and brain stem, while in the peripheral tissues, its expression is reported in skeletal muscle, skin, GI tract, cardiomyocytes, adrenal gland, and blood cells (Chen et al. 2004). In the brain, UCN3 is expressed in the hypothalamus. It is also expressed in the heart, skeletal muscle, small intestine, pancreatic β-cells, renal cortex, adipose tissue, and placenta (Deussing et al. 2010; Jamieson et al. 2006; Kavalakatt et al. 2019a; Pepels et al. 2010a; Rademaker et al. 2006; Roustit et al. 2014b; van der Meulen et al. 2015; Takahashi et al. 2004). UCN3 is an established marker for mature functional β-cells (van der Meulen et al. 2012) and the main endogenous ligand of CRFR2 (Li et al. 2002). Urocortin has also been reported to have autocrine and paracrine functions (Alarslan et al. 2020; Borges et al. 2015; Hashimoto et al. 1993; Kavalakatt et al. 2019a; Li et al. 2013).

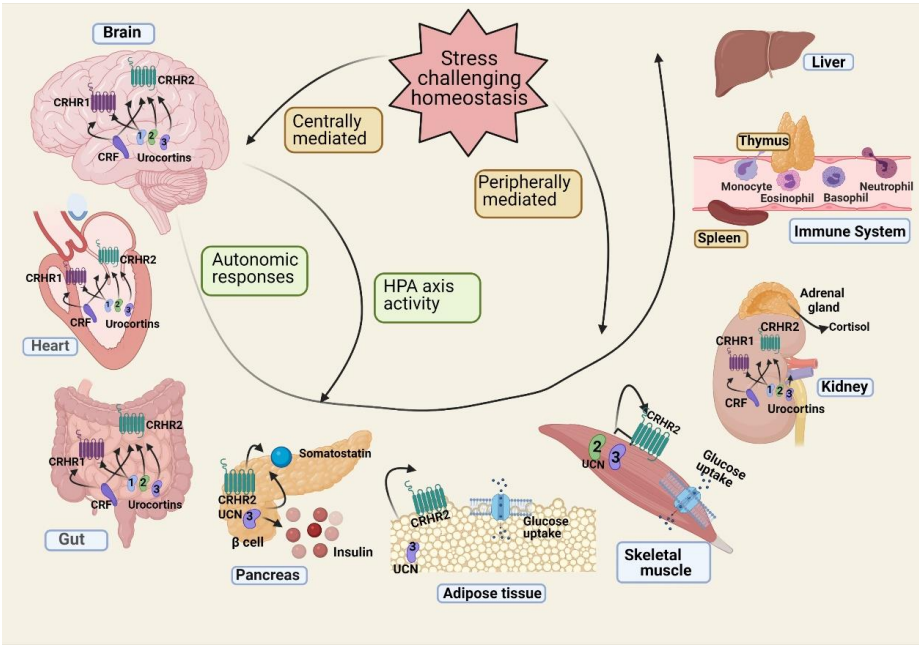

Figure 2: Central and peripheral expression of the members of the CRF family and the interactions of the ligands with their receptors. *The CRF family consists of CRF, UCN1-3, and two receptors, CRFR-1 and CRFR-2. They are expressed centrally and in the peripheral tissues, including the brain, heart, gut, pancreas, adipose tissue, skeletal muscle, kidney and immune system. Under conditions challenging stress homeostasis, HPA is activated, and CRF stimulates cortisol secretion, the main glucocorticoid, via ACTH. In addition, autonomic activity is modulated. Peripherally, UCN3 is co-released with insulin from β-cells and induces feedback inhibition of insulin secretion through modulation of somatostatin expression. UCN3 also improves glucose uptake in adipose tissue and skeletal muscle. Created with Biorender.com*

While UCN1 and CRF bind to both receptors CRFR1 and CRFR2, UCN2 and UCN3 bind specifically to CRFR2 (Lewis et al. 2001; Reyes et al. 2001). CRFR1 is expressed mostly in the cerebral cortex and anterior pituitary lobe, whereas CRFR2 is expressed in the central system and abundantly in the peripheral tissues (Valdez et al. 2003). CRF has a higher affinity to CRFR1 than CRFR2, whereas UCN1 has similar binding affinities to both receptors. On the contrary, UCN2 and UCN3 bind specifically to CRFR2, and UCN3 shows the highest specificity to CRFR2 (Valdez et al. 2003) (Figure 3). There are conflicting reports about activation and inhibition of

the HPA-axis through CRFR-2. While some studies report the decreased activity of the HPA axis with CRFR2 activation, others demonstrate that injection with CRFR2 agonists UCN2 and UCN3 stimulates the activity of the HPA axis (Valdez et al. 2003; Jamieson et al. 2006; Maruyama et al. 2007); this suggests species and stress-specific regulation of the HPA axis by CRFR2.

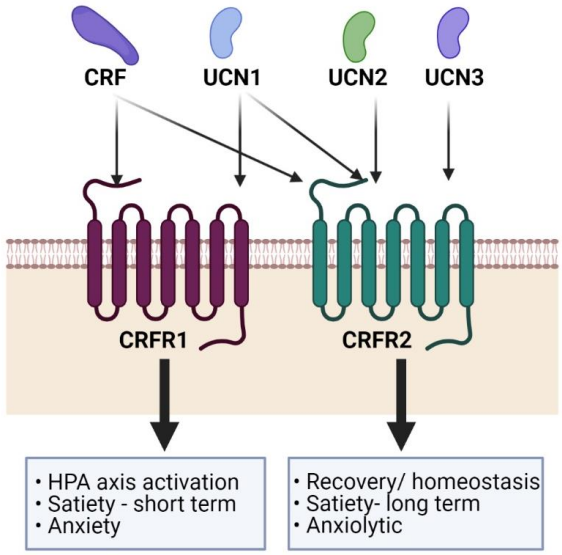

Figure 3: CRF receptors and their interaction with the peptides CRF and UCN1-3. *The figure represents the interaction of peptides with their respective receptors. While CRFR-1 activation results in stimulation of the activity of the HPA axis, anxiety, and short-term satiety, CRFR-2 activation leads to long-term satiety and is responsible for stress recovery and homeostasis. (Created in Biorender.com)*

These neuropeptides bind to CRF binding protein (CRF-BP), a secreted glycoprotein that binds with high affinity mainly to CRF (Orth and Mount 1987). Mature CRF-BP protein lacks transmembrane domains and phosphatidyl-inositol signal. The role of CRF-BP is unclear since results from studies have been contradictory. It has been suggested to reduce CRF receptor activity and peptide bioavailability by binding to the ligand and facilitating enzymatic degradation (Behan et al. 1995). On the other hand, CRF-BP could also protect the peptides from degradation during diffusion to membrane-bound CRF receptors (Kemp, Woods, and Lowry 1998). CRF-BP has been hypothesized to have signaling roles depending on

the bound ligand (Chan, Vale, and Sawchenko 2000). The level of CRF-BP sequestration to UCNs reflects a physiologic reservoir that another ligand could competitively release with varied pharmacological effects. Hence, the degree of binding between CRF-BP and UCNs could be of metabolic importance. UCN1 binds to CRF-BP with comparable affinity as CRF; however, it dissociates twice as slowly (Henriot, Dautzenberg, and Kilpatrick 1999). On the contrary, UCN3 does not bind to CRF-BP appreciably, while murine UCN2 binds with moderately high affinity(Boorse et al. 2005).

The major setback in using these secreted peptides as potential therapeutic agents is their short half-life (Patel et al. 2012). While UCN1 has a comparably higher half-life of 2.9 h, the half-lives of UCN2 and UCN3 are 15.7 min and 4.4 min, respectively. Hence, gene transfer and preparation of stable peptides are being considered (Giamouridis et al. 2018a).

2.3 Signaling pathways of CRF family neuropeptides

CRFRs belong to the secretin receptor family of G protein-coupled receptors (GPCRs) activated by peptide ligands. Upon binding with the peptide ligands, the CRFRs undergo structural alterations and couple with G-proteins activating intracellular signal transduction pathways (Figure 4). The CRF family achieves its physiological roles primarily through the activated adenylyl cyclase cyclic adenosine monophosphate (cAMP) signaling pathway (Rossant et al. 1999). Increased intracellular cAMP and Ca^{2+} levels activate kinases, including protein kinase A (PKA) and calcium/calmodulin-dependent kinase II (CaMKII). cAMP releases the catalytic subunit of PKA, which phosphorylates target proteins such as Ca^{2+}/ cAMP response element-binding protein (CREB). CREB binds to cAMP response elements (CREs), *c-fos,* or macrophage migration inhibitory factor (Brar, Jonassen, Egorina, Chen, Negro, Perrin, Mjøs, et al. 2004) after phosphorylation.

The biological effects of the CRF family are also achieved through activation of phosphatidylinositol-3-kinase (PI3K), protein kinase B/AKT, mitogen-activated protein kinases (MAPK), PKA, along with other less clearly defined alternative signaling pathways. The binding of UCNs to their receptors activates the PI3K/AKT pathway, which inhibits apoptotic proteins caspase 9, Bad, Bax, and Bcl2, thereby

directly affecting the intracellular equilibrium of the Bcl2 protein family and hence, enhancing cardio-protection (Onorati et al. 2013).

The MAP kinase signaling pathway is activated through extracellular signal-regulated kinase (ERK1/2) and exchange proteins activated by cAMP (EPAC). In addition, the PI3K-AKT pathway is activated with PKA regulating Sarco/endoplasmic reticulum calcium ATPase (SERCA). CRFR2 activation leads to elevated expression of inducible nitric oxide synthase (iNOS). Endothelial NO synthase (eNOS) is also activated through cAMP- and CaMKII-dependent pathways leading to increased Ca^{2+} influx.

Other signaling pathways of CRF neuropeptides also include AMPK and NFκB pathways (Adão et al. 2015). The vast range of signaling pathways impacted by the CRFergic system highlights its importance in mediating biological effects through several tissues. CRF was demonstrated to induce a thermogenic effect in skeletal muscle by activating AMPK and PI3K pathways (Solinas et al. 2006). UCN3 overexpression mediated increased glucose uptake by activating PI3K-AKT, ERK, and AMPK signaling pathways (Roustit et al. 2014b). Further, UCN2 has also been reported to increase glucose uptake in the heart through activating the AMPK pathway (Li et al. 2013). In cardiomyocytes, interleukin-6 (IL6) release was stimulated in an NFκB-dependent manner through CRFR2. UCN1 also induces IL6 release through cAMP accumulation and activation of PKC and MAPK signaling cascades.

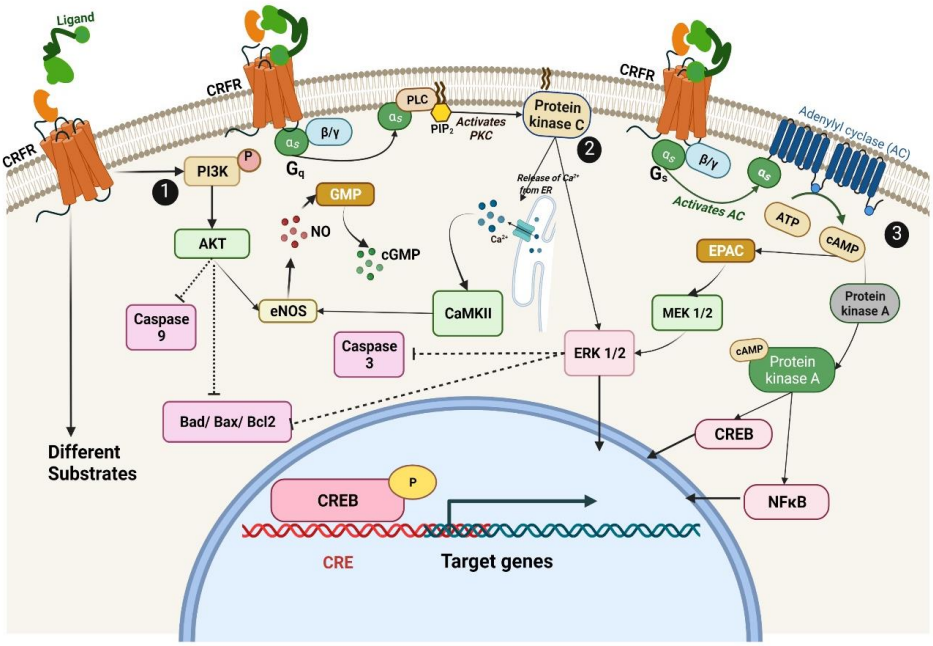

Figure 4: Representation of signaling pathways activated after binding of UCNs to CRFRs. *Binding of CRF or UCNs to the receptors, with the N-terminal segment integrated into the transmembrane domains, activates the receptor, and binds to G-proteins. 1) This catalyzes phosphorylation and activation of the PI3K-AKT signaling pathway, which in turn also inhibits apoptotic proteins caspase 9, Bad, Bax, and Bcl2. Both cAMP-PKA and PI3K-AKT pathways phosphorylate eNOS resulting in marked cellular NO production stimulating cGMP signaling. 2) Alternatively, CRFR binding with G_q activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG); this activates protein kinase C (PKC), which stimulates the release of Ca^{2+} from the endoplasmic reticulum activating CaMKII. PKC also activates MAPKs, including ERK ½. 3) The primary pathway activated is the cAMP pathway. The coupling of G proteins with CRFRs stimulates the conversion of ATP to cAMP, which activates two cascades - the MAPK/ERK pathways and phosphorylation of the CREB transcription factor. The increased cAMP concentration stimulates PKA and EPAC; this leads to posttranslational modification of target proteins, including ERK ½, CREB, and NFκB. All the alternate pathways lead to transcription of target genes and CRE genes, triggering various biological and physiological responses. Created in Biorender.com*

2.4 Physiological roles and functions of CRF family neuropeptides

2.4.1 Mood and behavior

The CRF pathway regulates the HPA axis, and the hypersecretion of CRF along with disrupted downstream pathways of CRF is strongly associated with mental disorders (Gallucci et al. 1993). The disrupted HPA axis has vital role in many mental disorders such as anxiety and depression. It is postulated that while CRF/CRFR1 mediates the initial stress reaction, UCN/CRFR2 modulates the late phase of the adaptation (Kozicz 2007; Reul and Holsboer 2002; Kozicz, Sterrenburg, and Xu 2011). UCN2 knock-out mice showed altered social behavior of reduced aggressiveness and passive behavior (Breu et al. 2012).On the contrary, UCN2 administration in mice showed anxiolytic and anti-depressive behaviors depending on the UCN2 fragment sequence that was administered, this effect being mediated with N-terminal sequence (Bagosi et al. 2018). Analogs and mimetics of UCN3 had anti-depressive effects similar to the original UCN3 peptide (Rákosi et al. 2014).UCN3 neurons in the hypothalamus were activated and associated with defensive behavior to threat stimulus, the ablation of which altered this behavior pattern (Horii-Hayashi et al. 2021). However, overexpression of UCN3 in the perifornical area nucleus elevated anxiety-like patterns, and UCN3 inhibition suppressed this behavior (Kuperman et al. 2010; Anthony et al. 2014). Also, UCN3 deficient mice did not show any HPA activity or depression-like behavior (Deussing et al. 2010). Some experimental studies have suggested that the manipulation of CRFR2 is affected by cell type, dose, duration, and experimental techniques used to influence results obtained (Hauger et al. 2006). Hence, although CRFR2 activation could act as a negative control of anxiety due to CRFR-1 activation, the desired CRFR2 mediated responses can be achieved through accurate control of response time and initial CRFR1 activation.

2.4.2 Food intake

Fasting is presumed to reduce satiety factors while feeding would have the opposite effect. Hence, different fasting conditions have been tested to study the CRF expression in response to feeding, fasting, and re-feeding in animal models (Rønnestad et al. 2017). UCN2 injection decreased food intake, reversed with CRFR2 antagonist Astressin-B (Martínez et al. 2002). UCN2 administration also induced satiety and, at low doses, did not affect signs of malaise (Fekete and Zorrilla 2007; Zorrilla et al. 2004). Although UCN2 deficient mice show normal food intake, UCN2 deficiency attenuates anorectic effects of fenfluramine, indicating a downstream role in serotonin's satiety effects (Chen, Zorrilla, et al. 2006). Central UCN3 injection diminished food intake and gastric emptying in mice, rats, and *Xenopus africanus* (Ortega, Lovejoy, and Bernier 2013; Boorse et al. 2005). Furthermore, peripheral injection of UCN3 in Siberian sturgeon reduced food intake (Zhang, Wu, et al. 2016). UCN3 was found to interact with thyrotropin-releasing hormone (TRH) to regulate food intake through synaptic interactions in the proopiomelanocortin (POMC) neurons of mice and rats (Péterfi et al. 2018). Intracerebroventricular (ICV) administration of UCN3 also reduced food intake in both lean and obese mice (Ushikai et al. 2011).

Studies have found a strong association between CRF peptides, anxiety, and feeding behavior in the past decade. Antagonists to CRFR1 and CRFR2 have revealed decreased anxiety, depression, and stress-induced anorexia. Studies have reported that antalarmin and CP154526, antagonists for CRFR1, reduced anxiety and depression-like behavior (Bale and Vale 2004a). Further, CRFR2 antagonist antisauvagine-30 decreased stress-related anorexia (Sekino et al. 2004; de Gortari et al. 2009). Hence, the members of the CRF family promise therapeutic targets to treat eating disorders, anxiety, and depression.

2.4.3 Energy balance and metabolism

Bodyweight gain and increase in the amount of WAT occur when the energy intake is excessive compared with expenditure. Brain circuitries that integrate central and peripheral signals play a critical role in maintaining energy homeostasis. Molecules including leptin, resistin, Plasminogen activator inhibitor-1 (PAI-1), visfatin, retinol-

binding protein (RBP4), and corticosteroids can regulate energy storage and fat mass (Trayhurn and Rayner 1996).

Obese animals react quickly to food deprivation and stress stimuli which strongly elicit the CRF system even in genetically induced obese animals. Administration of CRF and CRF-(6-33) fragment prevented weight gain in obese rats with no effect on the lean rats suggesting a possible dysregulation of the CRF system in obesity (Rohner-Jeanrenaud et al. 1989; Heinrichs et al. 2001). Furthermore, CRFR2 mRNA levels were decreased in obese rodents (Richard et al. 1996). Leptin has been known to reduce HPA activity, and with obesity, leptin was shown to reduce CRF in the paraventricular hypothalamic nuclei (Ahima et al. 1996; Giovambattista et al. 2000). The CRF system has been established to reduce energy storage through reduced food intake and augmented energy expenditure. The anorectic effects of CRF with exercise, estradiol, and caffeine have been well established along with their association with the thermogenic actions of serotonin receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Rivest and Richard 1990; Dagnault, Ouerghi, and Richard 1993; Racotta, Leblanc, and Richard 1994; Bovetto, Rouillard, and Richard 1996); this indicates that CRF can play a role in energy balance. CRF is reduced in the obese state, which is demonstrated with a reduction of body weight with CRF administration in the brain (Arase, Shargill, and Bray 1989). It has been established that adrenalectomy could reverse genetic obesity, and it reduces food intake in obese, diabetic mice and fatty rats via an increase in CRF expression, which may reverse genetically prone obesity.

UCN2 and CRFR2 knock-out mice were protected from HFD induced obesity and had improved glucose tolerance and insulin sensitivity (Chen, Brar, et al. 2006; Bale et al. 2003b). UCN3 transgenic mice were also protected from HFD induced obesity, had lower plasma glucose, and improved glucose disposal and glucose tolerance (Jamieson et al. 2011a). Even transient overexpression of UCN3 in skeletal muscle was shown to improve glucose metabolism and insulin signaling (Roustit et al. 2014a). UCN3 overexpression in the perifornical area of the brain increased energy expenditure (Kuperman et al. 2010).

2.4.4 Pancreatic hormone secretion and glucose homeostasis

Pancreatic islets play a critical role in maintaining glucose homeostasis through the secretion of the two major hormones, insulin and glucagon. These hormones are secreted by the α- cells (glucagon) and β-cells (insulin) of the pancreas. In addition, other peptides are also secreted from the α- and β-cells, such as ghrelin, neuropeptide Y (NPY), CRF, UCN3, and glucagon-like peptide-1 (GLP1) (Volante et al. 2002; Myrsén, Ahrén, and Sundler 1995; Date et al. 2002; Petrusz et al. 1983). CRFR1 and CRFR2 have also been reported in pancreatic islets suggesting paracrine/autocrine effects of the CRF and Urocortins through the receptors (Kanno et al. 1999a). Although glucose has been reported to induce glucagon secretion, the signals from the autonomic nervous system are the main mechanism responsible for the hypoglycemia-induced glucagon secretion (Taborsky 2011; Taborsky, Ahrén, and Havel 1998).

Crynda *et al.* have reported increased CRF, UCN2, and UCN3 mRNA expression in the placenta with gestation in mice (Drynda et al. 2018). This increase was associated with β-cell adaptation in pregnancy (Rieck and Kaestner 2010). Circulating UCN2 levels are also increased with pregnancy, suggesting a possible role of UCN2 in β-cell responses. Furthermore, blocking CRFR2 agonist-induced mild glucose intolerance did not progress into gestational diabetes; hence the peptides act together to maintain cell adaptations (Simpson et al. 2020a). Circulating CRF is also upregulated as pregnancy progresses in humans (Campbell et al. 1987). UCN3, along with gamma-aminobutyric acid (GABA), is secreted from the β-cell, which regulates somatostatin secretion. GABA neurotransmitters also modulate GLP1 and glucagon and stimulate somatostatin secretion from pancreatic delta cells. UCN3 is released by beta cells (in rodents and humans) and by alpha cells (in other primates) which act as a part of the feedback loop regulating insulin secretion via the effects of UCN3 on somatostatin secretion (van der Meulen et al. 2015; Li et al. 2003). UCN3 has a more potent effect than CRF on insulin secretion (Li et al. 2003). The stimulation of glucagon and insulin secretion by UCN3 is also observed *in vitro* using rat islets, and this is blunted with pre-treatment with a CRFR2 antagonist, Astressin 2-B (Ast2B) (Rivier et al. 2002). UCN3 is also a marker for functional β-cells and delineates β-cell maturation (van der Meulen et al. 2012).

Various studies have revealed the complex role of UCN3 in glucose homeostasis. UCN3 knock-out (KO) mice have been shown to have lower basal insulin and blood glucose levels compared to wild-type mice (WT). They also do not develop glucose intolerance and liver dysfunction as WT mice do. Further, aged UCN3 KO mice have improved glucose homeostasis compared with age-matched WT mice (Li et al. 2007). Hence, overall UCN3 knock-out seems to be associated with a favorable metabolic effect. On the other hand, UCN3 overexpression shows protection against metabolic disorders, leading to reduced blood glucose, improved glucose tolerance, and maintenance of lean body composition when mice are fed with HFD. Also, $UCN3^{+}$ mice have augmented energy intake in the basal state (Jamieson et al. 2011b). Thus, UCN3 in the brain and periphery contributes to transgenic animals' metabolic balance.

2.5 Genetically tailored animal models unravel the role of CRF family neuropeptides in physiology

The physiological significance of CRF and UCNs has been studied previously using various animal models, summarized in Table 1. It has been generally observed that role of CRF in the brain is area specific. Overexpression of CRF can reduce stress-induced anxiety or increase depression-like symptoms depending on the area-specific overexpression. However, pups from homozygous $Crf^{-/-}$ mice do not survive due to adrenal gland dystrophy and lung dysplasia (Muglia et al. 1995). On the contrary, CRF overexpression resulted in symptoms similar to Cushing's syndrome with increased corticosterone levels (Stenzel-Poore et al. 1992). Overall, CRF overexpression or KO impaired stress, behavior, and immune responses.

CRFR2 KO animals showed more anxiety than CRFR1 knock-out models (Bale and Vale 2004b). The significance of the CRF receptors was further investigated in animal models of its ligands UCN1,2 and 3. Animals models were further crossbred, enabling double and triple KO models of CRFR1/ CRFR2 and UCN1/UCN2/UCN3, respectively (Neufeld-Cohen, Evans, et al. 2010b; Neufeld-Cohen, Tsoory, et al. 2010b). Interestingly, double knock-outs demonstrated reduced stress levels compared to the triple KO models. However, triple KOs could not recover from stress even after 24 hours, implying that the UCNs are critical in stress recovery. Janssen *et al*.

suggested that in response to stress CRF system is activated in the brain and neuron-specific areas to mediate environmental condition-specific adaptations (Janssen and Kozicz 2013).

UCN3 null mice exhibited altered metabolic and energy homeostasis. However, no change in anxiety or depression-like symptoms was observed in another UCN3 null strain (Li et al. 2007). UCN3 overexpression in the brain resulted in increased stress-related behavior in non-restraint mice. Nevertheless, exposure to acute stress did not significantly change between mice with overexpression or WT mice. In line with this, Kuperman *et al.* showed that UCN3 overexpression induced anxiety behavior in a site-specific manner (Kuperman et al. 2010). $UCN3^{+}$ mice exhibited a metabolically favorable phenotype with enhanced skeletal muscle mass, increased glucose disposal, protection against HFD-induced obesity, and hyperglycemia (Jamieson et al. 2011b). Short-term local overexpression of UCN3 in the skeletal muscle could also enhance glucose uptake and activate insulin signaling in mice (Roustit et al. 2014b). $UCN3^{-/-}$ mice showed that UCN3, similar to UCN2 and UCN1, played a role in regulating glucose homeostasis. Double and triple knock-out mice revealed the critical role of these factors in stress recovery (Neufeld-Cohen, Evans, et al. 2010a; Neufeld-Cohen, Tsoory, et al. 2010a). In these knock-out models, the effect on other organs has not been reported. Hence, there may be impairments in cardiac, liver, kidney, pancreas, skeletal muscle, and reproductive functions. Overall, the assessment of UCNs and CRFR2 genetic animal models support the role of CRFR2 in anorexigenic responses, metabolic functions, and regulation of specific stress-related responses that complement CRFR1 and CRF. Recent studies have also highlighted the role of UCNs and especially UCN3 in regulating glucose and energy homeostasis.

Table 1: Characteristics of genetic mouse models targeting CRF family of peptides and their receptors

Genotype	Genetic Modification	Phenotype and Characteristics
CRF overexpression (OE) and knockout (KO)		
CRF-OE^{Mtl}	OE of rat CRF driven by *Metallothionine 1* promoter (Stenzel-Poore et al. 1992)	Decreased motility and attention, elevated anxiety and corticosterone, Cushing-like phenotype

CRF OE$^{Thy1.2}$	OE of rat CRF driven by *Thy-1* promoter (Dirks et al. 2002)	Decreased locomotion, anxiety, reactivity, and habituation. Elevated corticosterone, slightly increased ACTH, Cushing-like phenotype at 6 months.
CRF-COE^{CNS} CNS specific	Conditional OE of rat CRF driven by *Rosa26* promoter and induced through *Nestin-Cre* (Lu et al. 2008)	Hypersecretion of corticosterone under stress, decreased motility and increased REM sleep
CRF-COE^{Del}	Conditional OE of murine CRF driven by *Rosa26* promoter and induced through *Deleter-Cre (Dedic et al. 2012)*	Increased body weight, NREM sleep, and corticosterone levels at circadian trough, Cushing-like phenotype.
CRF$^{-120/+}$	Gain of function mutation in the CRF promoter region (Bentley et al. 2014)	Increased obesity and corticosterone levels, hyperglycemia, hyperinsulinemia, and muscle wasting
CRF-*KO*	Replaced CRF coding region (Muglia et al. 1995)	Reduced HPA axis activity, decreased stress-induced and basal corticosterone levels, increased anxiety, locomotion, and learning
UCN1 knockout		
UCN1-*KO*	Replaced coding region (Vetter et al. 2002)	Impaired HPA axis activity and increased anxiety
UCN1$^{-/-}$	Replaced exon region (Zalutskaya et al. 2007)	Reduced HPA adaptation to recurrent stress
UCN2 overexpression (OE) and knockout (KO)		
UCN2-COE Conditional	Conditional OE driven by *Rosa26* Promoter and induced through *SF-1-Cre (Spyroglou et al. 2015)*	Decreased adrenal and ovarian steroidogenesis
UCN2-KO	Replaced exon region (Chen, Zorrilla, et al. 2006)	Increased anxiety, nocturnal ACTH, and corticosterone levels
UCN2$^{tz/tz}$	Replaced open reading frame (Breu et al. 2012)	Reduced aggression and passive social interaction in male mice
UCN3 overexpression (OE) and knockout (KO)		
UCN3OE	OE of mouse UCN3 driven by *Rosa26* promoter (Jamieson et al. 2011b)	Protected against diet-related obesity, lean body composition, reduced ACTH levels following stress, increased anxiety and altered serotonin levels.
UCN3-KO	Replaced coding region (Li et al. 2007)	Decreased glucose-induced insulin secretion, basal glucose, and insulin secretion under high-fat diet. Increased food and ethanol intake.

UCN3$^{tz/tz}$	Replaced open reading frame (Deussing et al. 2010)	No change in anxiety, HPA axis activity, immobility, and social discrimination abilities.
CRFR-1 knockout		
CRFR-1-KO	Replaced exon region 8-13 (Timpl et al. 1998)	Decreased HPA axis activity with lowered corticosterone levels at baseline and with stress. Reduced anxiety and fear memory consolidation
CRFR-2 knockout		
CRFR-2-KO	Replaced exon region 10-12 (Bale et al. 2003a)	Decreased ACTH and corticosterone levels in response to stress. Increased anxiety, food intake and high fat diet food intake
Double (dKO) and Triple knockouts		
CRF-R1/CRF-R2 dKO	Crossbreeding (Bale et al. 2002)	Reduced HPA stress response and anxiety only in females
UCN1/UCN 2-dKO	Crossbreeding (Neufeld-Cohen, Evans, et al. 2010b)	Elevated HPA response only in males and decreased anxiety
UCN1-KO/UCN2-KO/UCN3-KO	Crossbreeding (Neufeld-Cohen, Tsoory, et al. 2010b)	No change in HPA activity, reduced locomotion and increased anxiety following 24h of acute stress

2.6 Role of CRF family neuropeptides in health and disease

2.6.1 Vascular system/Vasodilation

The vascular system consists of the cardiovascular and lymphatic systems in mammals. It plays a critical role in immune responses, cell and tissue fluid homeostasis, and functions as an important component of other systems such as the respiratory system, digestive system, kidneys, and thermoregulation. All three Urocortins (UCN1-3) are associated with vasodilation, which is mainly mediated through the relaxation of vascular smooth muscle cells (VSMCs). The principal action is via CRFR2 with adenylyl cyclase activation and cAMP accumulation. These effects were significantly blunted with CRFR2 antagonists and inhibition of adenylyl cyclase, PKA, and P38 (Kageyama et al. 2003). UCN mediated effects were also severely attenuated in CRFR2$^{-/-}$ mice. CRFR2$^{-/-}$ mice were hypertensive, demonstrating the role

of UCNs in regulating blood pressure and vascular tone (Chen et al. 2003). Furthermore, when the CRFR2 receptor was pharmacologically blocked, there was a significant increase in arterial pressure and peripheral resistance in heart failure (HF) animals (Rademaker et al. 2005).

Renal arteries were reported to be vasodilated with UCN1 administration through cAMP production, activation of potassium channels, and increased synthesis and release of nitric oxide (NO) as well as Ca^{2+} (Rademaker et al. 2005; Sanz, Monge, et al. 2003). Intra-arterial infusion of UCN2 and UCN3 in normal humans causes a significantly prolonged vasodilation mediated through NO and cytochrome P450 metabolites of arachidonic acid (Smani et al. 2011). Urocortins could also impact blood pressure through modulation of various vasoactive systems such as angiotensin II (AngII), endothelin-1 (ET1), serum, and aortic NO, as well as angiotensin-converting enzyme (ACE) correlating with reduced systolic blood pressure (Rademaker et al. 2008; Yang, Liu, and Li 2010). In addition, UCNs can also modulate smooth muscles, as reported with UCN3 induced broncho-relaxation in a mouse model of airway inflammation blunted with CRFR2 antagonists. However, the UCN3 modulated relaxation was not affected by epithelium removal or inhibition of NO and cyclooxygenase (Moffatt, Lever, and Page 2006).

UCN-induced NO production from endothelial cells functions to inhibit the pro-inflammatory cytokines, chemokines, adhesion molecules, production of tissue factor, and aggregation of platelets. On the other hand, endothelial factors such as TNFα, IL8, and interferon-gamma (IFNγ) also induce the secretion of UCNs (Borges et al. 2015). All UCNs enhanced the levels of NO along with antioxidant enzymes superoxide dismutase and glutathione peroxidase (Liu et al. 2005).

Studies have shown that UCNs promote mitogenic responses and hypertrophy in the heart, including increased protein synthesis, sarcomere rearrangement into striated form, and induction of β-myosin heavy chain (β-MHC) (Davidson et al. 2005). Among the UCNs, UCN3 had the most potent effect followed by UCN1 and UCN2 through mostly activation of PKA, PI3K/AKT, increased expression of transcriptional co-activator P300, and glycogen synthase kinase (GSK3β), factors responsible for cardiac

remodeling (Walther et al. 2014). Taken together, the CRF family of neuropeptides are unique modulators of the vascular system with autocrine and paracrine functions.

2.6.2 Cardiac protection

UCNs and their receptors are highly expressed in the cardiovascular system eliciting a wide range of pathways in multiple targets. They have demonstrated several functions, including vasodilation and anti-inflammatory roles. The experimental heart failure model in sheep by Rademaker *et al.* showed dose-dependent cardiac function improvement (Rademaker et al. 2002) by UCN1. When heart failure was induced in sheep, administration of UCN1 induced a range of beneficial effects such as increased urine and sodium excretion, creatinine excretion, and cardiac output. It was also able to decrease plasma vasopressin, renin, endothelin-1, and aldosterone levels.

UCNs possess cardioprotective functions through improved cell survival in isolated cardiomyocytes and isolated perforated rat heart, exposed to simulated hypoxia and ischemia/reperfusion (I/R) injury (Okosi et al. 1998; Townsend et al. 2007). These functions were abolished in CRFR2 null mice, making the mice more susceptible to I/R injury (Brar, Jonassen, Egorina, Chen, Negro, Perrin, Mjøs, et al. 2004). Following I/R injury, all three UCNs reduce free radical activity. Microarray analysis in rat coronary occlusion model pre-treated with UCN1 or -2 before the reperfusion injury revealed several UCN regulated genes responsible for multiple response pathways. These included oxidative stress, apoptosis, and metabolism. GPCR signaling modulated these genes through, and several of these genes were also related to MAPK activation (Barry et al. 2010). Overall, the CRF family of neuropeptides could be potential targets for treating hypertension, heart failure, and coronary heart disease. Furthermore, UCNs also augment cardiomyocyte contractility mediated through increased intracellular calcium concentration (Kanno et al. 1999b). A broader understanding of the receptor-ligand interaction and sexual dimorphism is essential to unravel the underlying mechanisms and develop better therapies.

2.6.3 Sleep apnea

Obstructive sleep apnea (OSA) syndrome is characterized by reoccurring complete or partial upper airway obstruction during sleep ('Standards and indications for cardiopulmonary sleep studies in children. American Thoracic Society' 1996). OSA is associated with hypertension, insulin resistance (especially with obesity), inflammation, and morbidity in both children and adults. Several animal and human studies have shed light on the stimulatory effect of sleep deprivation on the HPA axis. There is a bi-directional relationship between disturbed sleep and over-activation of the HPA axis mediated through cortisol levels (Meerlo et al. 2002; McEwen 2008; Guyon et al. 2014). Additionally, the elevated cortisol levels cause a circadian misalignment of glucocorticoid levels, a powerful synchronizer of the circadian system, leading to various long-term adverse events (Oster et al. 2017). Recurrent sleep deficit results in elevated CRF levels, blunted response to CRF, and impaired glucocorticoid recovery in rats (Guyon et al. 2014) and humans (Guyon et al. 2017). Interestingly, Vgnotzas *et al.* have shown that obesity, characterized by a low 24hr mean cortisol levels and mediated by hyposecretion of CRF, could dispose obese people to sleep apnea (Vgontzas et al. 2007).

Intermittent hypoxia exposure was shown to reduce CRFR1 levels but increased CRFR2 levels and subsequently activated CRFR2 signaling pathways, suggesting a possible adaptive defense mechanism (Wang, Nguyen, and Mifflin 2018). Snow *et al.* proposed that UCNs were increased with sleep apnea (Snow et al. 2010). It was also shown through studies from Gozal *et al.* that UCN3 in urine was significantly increased with pediatric OSA (Gozal et al. 2009). A US patent (US Patent No.9435814 B2) was issued for UCN3 as a biomarker to detect OSA and is being developed as a panel for pediatric OSA detection from urine samples. Taken together, the CRF family of neuropeptides could be potential biomarkers and targets in the diagnosis and treatment of OSA.

2.6.4 Diabetes and related complications

HPA axis activation has been associated with diabetes with and without other morbidities such as obesity and depression. This activation is correlated with increased ACTH and cortisol. In diabetic animals, CRF secretion is significantly increased.

However, there is also a decrease in CRF receptors in the anterior pituitary. Hence, this results in reduced ACTH response to CRF (SCRIBNER et al. 1991).

Diabetic nephropathy (DN) is one of the complications of diabetes resulting in kidney failure and chronic kidney disease. Vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGFβ1) are the main markers utilized for the progression of DN (Cooper et al. 1999). UCN1 inhibits TGFβ1, VEGF, and connective tissue growth factor (CTGF) induced by high glucose levels, decreasing extracellular matrix (ECM) accumulation. UCN1 was also able to improve creatinine clearance (Ccr) and albuminuria in DN rats. The pathological changes related to DN, including ECM accumulation indicated by the increased basement membrane width and the mesangial matrix expansion, and enhanced expression of TGFβ1 and VEGF1, was significantly reduced with UCN1 (Li et al. 2009). Sanz *et al.* have shown that UCN1 has a vasodilatory effect in the kidneys mediated by NO and activation of potassium channels. However, this vasodilation is diminished with diabetes in rats (Sanz, Fernández, et al. 2003).

In diabetes, UCN1 expression in the heart was markedly reduced, which correlated with increased apoptosis and cardiac dysfunction (Chen-Scarabelli et al. 2014b). Notably, UCN1 treatment attenuated cardiac dysfunction, inflammation, and cardiac fibrosis in rat diabetic hearts, although no effect on HbA_{1c} levels was observed (Liu et al. 2015). UCN2 gene transfer increased skeletal muscle insulin sensitivity, glucose-stimulated insulin secretion, reduced plasma glucose levels, diabetic retinopathy, and mortality in insulin-deficient Akita mice (Gao et al. 2020). UCN2 gene transfer also reduced hepatic glucose production, indicating an increased hepatic insulin sensitivity. UCN2 peptide increased glucose-stimulated insulin release from islets without causing any changes to somatostatin release, as earlier shown by UCN3 (Gao et al. 2020). A modified UCN2 peptide was developed that acts as an insulin sensitizer in skeletal muscle from obese mice (Borg et al. 2019). Furthermore, UCN3 expression has been reported to be blunted in diabetes in pancreatic β-cells and adipose tissue in mice, humans, and macaque monkeys (Kavalakatt et al. 2019a; van der Meulen et al. 2015). Moreover, increased plasma UCN3 levels were also associated with an increased risk of T2D (Alarslan et al. 2020). Li *et al.* have reported that under conditions of nutrient excess, UCN3 regulates insulin secretion (Li et al. 2007).

2.6.5 Inflammatory diseases

Inflammation is a key mechanism elicited in response to stress, infection, or injury. It is also the major characteristic of inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis. In a rat model of dyspepsia, the CRF signaling in mast cells regulates inflammation and the hypothalamus modulating anxiety-like behavior (Hagiwara et al. 2018). The severity of irritable bowel syndrome (IBS), with diarrhea as the dominant component, is associated with the CRF receptors present on mast cells (Hagiwara et al. 2019). The two receptors harbored on the mast cells have opposing immune responses. Macrophage function was regulated by CRF mediated through CRF receptor in lipopolysaccharides (LPS) induced cytokine production. Further, altered expression of CRF, UCN1, and UCN2 was observed in IBD patients. In male CRFR2 null mice, pancreatitis led to severe distortion in rough endoplasmic reticulum cisternae, unusual autophagic bodies, and mitochondrial swelling. These findings suggest that downregulation of CRFR2 occurs with the cellular stress-causing burden on ER and mitochondria (Kubat et al. 2013).

Both UCN1 and CRF expression levels were increased in intestinal macrophages of ulcerative colitis patients suggesting a pro-inflammatory role (Saruta et al. 2004). The pro-inflammatory role of UCN1 is further supported through stimulation of IL1β and IL6 production in PBMCs (Kohno et al. 2001). UCN1 secretion is stimulated by LPS, angiotensin, ROS, and inflammatory cytokines (Ikeda et al. 2009). It is secreted from the heart under stress conditions such as I/R-injury or heart failure (Wright et al. 2009).

UCN2 diminished pancreatitis and inflammation through the NFκB pathway of activated B cells (Yuan et al. 2019). There was a decrease in infiltrated immune cells in the pancreas with UCN2 administration. UCN2 induced macrophage apoptosis in a dose-dependent manner, exerting an anti-inflammatory action (Tsatsanis et al. 2005b). UCN3 expression levels were significantly reduced in the rat model of colitis. Also, knockdown studies of the CRF receptors suggested that their balanced expression is required to regulate UCN3 during inflammation and at basal levels (Mahajan et al. 2014).

2.7 Therapeutic potential and clinical development of CRF family neuropeptides

Several studies in recent years have highlighted the potential of the CRF system as a therapeutic target. The CRF receptors' activation in the brain and periphery can alter the functional consequences. Comparative studies of receptor agonists, including CRF and UCNs and antagonists, have been shown to mediate stress-related changes in various domains. The pharmaceutical industry has pursued to develop blood-brain barrier penetrating, CRF non-peptide antagonists, and CRF-specific ligands to exploit the neurobiological and physiological functionalities of the CRF system. Various clinical trials have been completed or are still ongoing.

One of the first suggestions for using the CRF system as a suitable treatment target has been depression and anxiety. Clinical and preclinical studies have implicated increased CRF levels and HPA activity in patients suffering major depression and anxiety through activation of CRFR1 receptor (Gallucci et al. 1993); this has led to the realization of several preclinical and clinical studies with CRFR1 antagonists. The first of such clinical studies was a phase-IIa trial to investigate the safety and efficacy of the NBI-30775/R121919 compound. This compound not only improved depression symptoms but also sleep parameters while not impacting the HPA axis activity. Despite these promising results, elevation in liver enzymes stalled further developments (Künzel et al. 2003; Held et al. 2004; Zobel et al. 2000). Another CRFR1 antagonist tested was NBI-34041 in a phase I study. This compound demonstrated safety, tolerability, and improved psychosocial stress parameters without affecting HPA axis regulation (Ising et al. 2007). Several CRFR1 antagonists underwent clinical trials such as CP-316,311, pexacerfont, ONO-2333Ms, SSR125543, GSK561679, and others; however, these treatments were unable to demonstrate any therapeutic effects to anxiety and therefore were discontinued (Binneman et al. 2008; Spierling and Zorrilla 2017). Nevertheless, the current view is that CRFR1 antagonists are effective in patients with dysregulated CRF systems. Hence, categorizing patients should be considered.

CRFR1 antagonism has been implicated in other stress-related pathologies such as the neurodegenerative disorder Alzheimer's disease (AD) (Zhang, Kuo, et al. 2016),

where R121919, a selective CRFR1 antagonist, showed promising results in AD mice. Treatment with the antagonist prevented cognitive impairments reduced cellular and synaptic impairments and beta-amyloid levels. Several other CRFR1 antagonists have been studied in clinical trials, such as Emicerfont - GW876008 for irritable bowel syndrome, Pexacerfont - BMS-562086 for alcoholism and anxiety, and Verucerfont - NBI-77860 - GSK561679 for congenital adrenal hyperplasia, alcoholism, and post-traumatic stress disorders. UCN1 has been implicated with therapeutic effects in collagen-induced arthritis (CAI), where UCN1 administration had markedly reduced incidence and severity in CAI. The therapeutic effects included abrogated swelling, cartilage bone destruction, and TH1 activated autoimmune and inflammatory response (Gonzalez-Rey et al. 2007).

Furthermore, CRFR1 antagonism has been implicated as a treatment option in eating disorders. CRA 1000, a CRFR1 antagonist, was shown to blunt stress-induced food intake (Hotta et al. 1999). All selective CRFR1 antagonists to date, including SSR125543, CP-154526, CRA1000, and antalarmin, have resulted in anxiolytic effects. Hence, these antagonists could be useful in treating obesity, especially associated with stress. In line with this, SSR125543 treatments in obese (*fa/fa)* Zucker rats reduced plasma glucose levels and insulin resistance (Doyon et al. 2007), suggesting an improvement in parameters related to diabetes. Furthermore, neurobiological advances highlight the contribution of the CRF system to the withdrawal and craving stages of the drug addiction cycle. In line with this, CRFR1 antagonists could have therapeutic potential in people who self-medicate emotional stress innately with disproportionate levels of drugs or alcohol (Koob and Zorrilla 2010); this is exhibited by the effect of CRFR1 antagonist on anti-drinking response in Marchigan-alcohol-preferring and isolation-reared-fawn-hooded rats (Zorrilla and Koob 2010). Obesity and drug addiction share behavioral and neural circuits, including distress trying to abstain from drug/food, inability to stop overeating/use, and dysphoria (Iemolo et al. 2013). These effects implicate the possible use of these antagonists in people who suffer emotional eating and addiction to palatable foods.

Upregulation of CRFR2 and translocation to membranes of serotonin neurons in the brain responsible for stress responses has been reported in Myalgic Encephalomyelitis / Chronic Fatigue Syndrome (ME/CFS), a chronic debilitating

disease; this activates acute stress response to minor stimuli leading to abnormal neuroendocrine, immune, and metabolic responses (Waselus et al. 2009). Therefore, treatment with receptor agonists to reverse this maladaptation has been proposed. Clinical trials using CT38, a CRFR2 agonist for ME/CFS, have been completed; however, the results are not yet available (Clintrials.gov). Furthermore, a patent was granted to the same group for the use of CRFR2 agonists such as CT38, UCN2, and UCN3 to treat maladapted stress-induced diseases such as ME/CFS. CRF agonist, Xerecept/ Corticorelin/ Achtrel, has been investigated in clinical trials for brain edema/tumor, cocaine dependence, Cushing's syndrome, and Dyspepsia. The results of these studies are not yet fully available.

Administration of peptide infusions of UCN2 and UCN3 have shown favorable effects in clinical heart failure; however, they are not suitable for chronic therapy due to their short half-lives; this was circumvented and sustained elevated levels of these peptides were obtained through gene transfer. *Giamouridis et al.* has demonstrated that UCN3 gene transfer could improve cardiac function of failing murine heart. This intrinsic improvement was suggested to result from enhanced Ca^{+2} handling and subsequent increase in systolic and diastolic function (Giamouridis et al. 2019). UCN2 gene transfer was suggested as a possible therapeutic target for type 1 diabetes as a single intravenous injection of the vector reduced blood glucose, increased skeletal muscle insulin sensitivity, glucose-stimulated insulin release, and plasma insulin levels. The improved insulin sensitivity and bioavailability enhanced glycemic indices in insulin-deficient murine models (Gao et al. 2020). UCN2 gene transfer also improved cardiac function in aged mice (Giamouridis et al. 2020) and restored dysregulation in liver metabolites due to HFD to normal levels, which could be the underlying mechanisms for improved glucose disposal and reduced insulin requirement (Kim et al. 2019b). UCN2, but not UCN3, gene transfer increased glucose disposal and reduced glucose levels. UCN2 and UCN3 impact differences could be due to disparities in the plasma levels of these peptides, the presence of peptidases, or possible receptor-independent effects (Giamouridis et al. 2018b).

Briefly, cumulative evidence highlights the role of the CRF family of neuropeptides in the pathophysiology of several stress-related and cardiometabolic

disorders. Therefore, further investigation on Urocortins, particularly the recently discovered UCN3, holds promising therapeutic potential.

3 Aims of the study

The main objective of the present study was to elucidate the role of UCN3 in obesity and diabetes.

The specific aims of the study were:

1) To assess the circulating UCN3 levels in obesity and type 2 diabetes in a cross-sectional adult population (publication I).
2) To evaluate the circulating UCN3 concentrations in pediatric obesity (publication II).
3) To identify the associations of UCN3 with metabolic markers of obesity and diabetes and to further investigate the effect of metabolic stressors on UCN3 levels (publications III and IV).
4) To study the effect of UCN3 on the signaling pathways of insulin, glucose uptake and ER stress, and heat shock response (publication IV).

4 Materials and methods

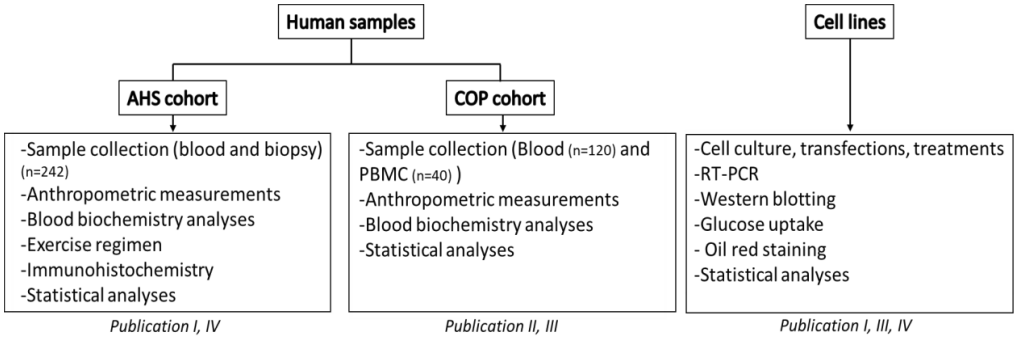

Figure 5: Flowchart of the study cohorts and the methods used in these studies

4.1 Study population, sampling, and blood biochemistry

4.1.1 Adult human study cohort (AHS) (Publications I and IV)

The AHS cohort consisted of adult male and female participants of 3 groups- normal-weight (20≤ BMI < 25 kg/m^2, n=37) and overweight/obese people (25 ≤ BMI≤ 40 kg/m^2) either without T2D (n=107) or with T2D (n=98). Written informed consents were obtained from all participants before their enrollment. The Institutional Review Board approved the study of the Dasman Diabetes Institute, and the principles of the Declaration of Helsinki were followed. People involved in regular physical activity within six months before the enrollment to the study or people with major diseases or use of medications unrelated to diabetes were excluded from the study. Participants were matched for age and sex during the selection process.

Data from the 144 non-diabetic participants, including 37 normal-weight and 107 overweight subjects from the AHS cohort, were analyzed in publication IV.

4.1.1.1 Exercise regimen and physical measurements

Eligible subjects (n=39, each overweight group) were enrolled in a monitored moderate exercise regimen at the Medical Fitness Centre (MFC) of Dasman Diabetes Institute. Before enrollment, each individual underwent initial fitness and physical

assessment. Dual-energy X-ray absorptiometry device (Lunar Radiation, Madison, WI) was used to measure whole-body composition. Each subject performed the Cardiopulmonary exercise test (CPET) (COSMED, Italy) using an electronically braked cycle ergometer. CPET assesses maximum oxygen consumption (VO_{2max}) and the maximum heart rate (max HR).

Grip strength and upper body strength were assessed using dynamometer and push-ups, respectively. Muscle strength and flexibility were further determined using sit-ups and forward bending tests. The exercise regimen included resistance training with cycling or treadmill and aerobic exercises of moderate intensity. Each session lasted 40 minutes at 65-80% max HR and included warming up and cooling down sections 10 min each at max HR of 50-60%. The subjects exercised 3 times per week for 3 months, and the recommended heart rates were maintained during exercise under the supervision of trained professionals at MFC. According to the exercise plan, strength training was performed two to three times per week. The intensity and duration of exercise for each session were recorded. The effectiveness of the exercise plan was assessed at the end of the program using the same fitness and physical stress test assessments performed before the enrollment.

4.1.1.2 Sample collection and analysis

Venous peripheral blood and subcutaneous adipose tissue biopsies were collected at baseline and following the three-month exercise program. PBMCs were isolated using the Ficoll-Hypaque density gradient centrifugation method, and plasma was separated using vacutainer tubes, aliquoted, and stored at -80°C. SAT biopsies of approximately 0.5 g were taken surgically after local anesthesia from the periumbilical area. Biopsies were quickly rinsed with cold PBS, divided into pieces, and stored at appropriate temperatures.

4.1.1.3 Blood biochemistry analysis

Hemoglobin A_{1c} (HbA_{1c}) levels were measured using Variant™ device (BioRad, USA). Concentrations of insulin (Mercodia, Sweden) and hs-C-reactive protein (hsCRP, Biovendor, USA) were measured using ELISA kits. Homeostatic model assessment of insulin resistance index (HOMA-IR) was calculated using the formula HOMA-IR $= \frac{(glucose\ x\ insulin)}{22.5}$. Lipid and glucose levels were measured using the Siemens Dimension RXL chemistry analyzer (Diamond Diagnostics, Holliston). All

assays were performed per the manufacturer's instructions. The human cytokine 27-plex panel kit and human 12-plex diabetes kit were used to measure the inflammatory and metabolic markers, respectively (Biorad, Hercules, CA). Fluorescence intensities were measured on the Bioplex-200 system, and analyte concentrations were determined using the Bioplex manager software (Bio-Rad).

Plasma levels of UCN3 (#LS-F12902, Lifespan Biosciences, USA), HSP60 (ADI-EKS-600, Enzo, PA, USA), and GRP78 (#ADI-900-214, Enzo LifeSciences, Switzerland) were assayed using ELISA kits. For UCN3 ELISA, the detection range is 0.156-10 ng/ml, the sensitivity was less than 0.156 ng/ml, intra-assay CV was <4.3% and inter-assay CV was <7.5%. Synergy H4 plate reader (Biotek, Winooski, VT, USA) was used to measure absorbance values.

4.1.2 Childhood Obesity Program (COP) cohort (Publications II and III)

The COP cohort consisted of 120 boys and girls with a mean age of 12 years, including normal weight (n=21), overweight (n=14), and obese children (n=85). A subset of this cohort was used for the PBMC transcriptomics study (study IV), including normal weight (n=8), overweight (n=10), and obese (n=22) children. Before enrollment, written informed consent was obtained from all children's parent(s). The study was approved by the Institutional Review Board of Dasman Diabetes Institute, Kuwait, and the principles of the Declaration of Helsinki were followed. Children were stratified based on BMI and percentile according to Centre for Disease Control (CDC) growth charts as follows: normal-weight (BMI/Percentile < 85^{th}), overweight ($85th \leq$ BMI/Percentile < 95th), and obesity (BMI/Percentile $\geq$ 95th) groups (Kuczmarski et al. 2002).

4.1.2.1 Anthropometric measurements

Standard physical examination by a pediatric endocrinologist was performed for all enrolled children, and the anthropometric measurements were taken according to the recommendations of the World Health Organization. Body fat percent and weight were measured using Pediatric Body Composition Analyzer/Segmental (GAIA KIKO Jawon Medical, South Korea) while the children wore light clothes and no footwear. Height measurements were taken using a stadiometer, and BMI was calculated per the

CDC growth charts. Children were grouped based on the cut-off points defined by Cole et al. (Cole et al. 2000).

4.1.2.2 Sample collection and blood analysis

PBMCs were isolated from blood and glucose levels, lipid profile, and HbA_{1c} levels were measured earlier in the AHS cohort. Insulin levels and liver function enzymes were quantified using Access 2 and AU480 Systems (Beckman Coulter, USA), respectively. HOMA-IR was calculated as above for the AHS group. C-peptide was measured using ELISA kits (Mercodia, Sweden).

Plasma levels of UCN1 (#LS-F6155, Lifespan Biosciences, USA), UCN2 (#LS-F39013, Lifespan Biosciences, USA), UCN3 (#LS-F12902, Lifespan Biosciences, USA), CRF (#LS-F5352, Lifespan Biosciences, USA), Spexin (#EK-023-81, Phoenix Pharmaceuticals, Burlingame, California, USA) were measured using ELISA. For UCN1 ELISA the detection range was 15.63-1000 pg/ml, the sensitivity <5.9 pg/ml, intra-assay CV <10% and inter-assay CV <12%. For UCN2 ELISA the detection range was 7.8-500 pg/ml, the sensitivity <3.1pg/ml, intra-assay CV <10% and inter-assay CV <12%. For UCN3 ELISA, the detection range was 0.156-10 ng/ml, the sensitivity <0.156 ng/ml, intra-assay CV <4.3% and inter-assay CV <7.5%. For CRF ELISA, the detection range was 12.35-1000 pg/ml, the sensitivity <4.91 pg/ml, intra-assay CV <10% and inter-assay CV <12%. For Spexin ELISA, the detection range was 0.11-1.07 ng/ml, the sensitivity 0.11 ng/ml, intra-assay CV <10% and inter-assay CV <15%. All assays were performed according to the manufacturers' instructions and the absorbance was measured using Synergy H4 plate reader (BioTek, USA).

Bead-based multiplexing Bio-plex 200 system was used for measuring Obesity and Diabetes markers. Obesity [Metabolism/Obesity 5-Plex Human ProcartaPlex™ Panel 1 (EPX09A-15804-901), Metabolism/Obesity 9-Plex Human ProcartaPlex™ Panel 2 (EPX09A-15804-901)] and diabetes (MILLIPLEX MAP Human Diabetes Panel, premixed 5-Plex Assay, HDIAB-34K-PMX5) panels were used. Fluorescence intensities were quantified with Bio-plex manager software v6 (Bio-Rad, Hercules, CA, USA). All assays were performed according to the manufacturer's instructions.

4.2 Immunofluorescence confocal microscopy (publication I)

Formalin-fixed, paraffin-embedded SAT sections were cut at 8µm thickness from the 3 groups of the AHS cohort (n=6 per group). The sections were deparaffinized, rehydrated, and subsequently followed by the antigen retrieval at pH 6 in a pressurized cooker, using DAKO reagents (Dako, Glostrup, Denmark). Endogenous peroxidase activity was quenched for 1 hour at room temperature (RT) using 3% H_2O_2. Blocking of non-specific antigens was done with 5% fat-free milk for 1 hour at RT, followed by 1% BSA for 1 hour. The sections were then incubated overnight with primary antibodies at 4°C. The primary antibodies used were anti-UCN3 (ab79121, Abcam, UK) and anti-adiponectin (#5901, BioVision, USA). Antibody specificity was assessed using negative control slides incubated without primary antibody or unspecific IgG. Slides were incubated with Alexa Fluor 488-conjugated rabbit secondary antibody (A-11008, Invitrogen, USA) for 1 hour at RT. Sections of the human pancreas were used as a positive control for UCN3 expression. Nuclear staining was done using 0.05% DAPI. Sections were imaged using Zeiss LSM710 confocal laser scanning microscope at 40X objective, and fluorescence intensities were quantified using ZEN software (Zeiss, Germany).

4.3 Cell culture, transfection, and treatments (publication I, III, IV)

Mouse pre-adipocyte (3T3-L1), mouse macrophage cells (RAW264.7), and human monocytic cells (THP-1) were purchased from ATCC (VA, USA). 3T3-L1 cells were cultured in DMEM media supplemented with 10% bovine calf serum (BCS). RAW 264.7 cells were grown in DMEM media with 10% fetal bovine serum (FBS), and THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS and 0.05 mM β-mercaptoethanol. 100 units/ml penicillin-streptomycin was added to all media.

THP1 cells, seeded at a density of 1 million cells/ml, were treated with high glucose (HG, 25 mM), palmitic acid (PA, 200 µM), or a combination of HG and PA

for a duration of 4 h and 24 h. Cells were harvested following treatments for RNA isolation and gene expression analyses.

Cells were grown to 100% confluence to differentiate 3T3-L1 pre-adipocytes to mature adipocytes. Cells were then initiated for differentiation (Day 0) using induction medium (culture media with 0.5 mmol/L isobutyl methylxanthine, 10 mmol/L insulin, and 0.25 mmol/L dexamethasone) following 2 days post-confluence. After 2 days of induction, media was changed to maintenance medium (culture media with insulin). For indirect co-culture of 3T3L-1 with RAW264.7, conditioned media from RAW264.7 cells were used as maintenance media. The latter was changed every 2 days until day 8 (D8). Differentiation was confirmed using lipid droplet staining with Oil Red O.

Transfection was done using the human UCN3 gene cloned into the pCMV6 vector with Myc-DDK tag (OriGene, Rockville, MD, USA). The control vector used was pCMV6 vector without UCN3, and Myc-DDK tagged (OriGene, Rockville, MD, USA). 3T3-L1 pre-adipocytes were transfected using lipofectamine LTX plus reagent (Invitrogen, CA, USA) with 2 μg of plasmid for 24 hours. Differentiated 3T3-L1 adipocytes were transfected using Cell Line Nucleofector Kit (Lonza, Basel, Switzerland). For electroporation, 2 × 106 cells were transfected with 2 μg of plasmid using Nucleofector program, A-033 for 24 hours. Following 24 hours of transfection, adipocytes were treated with 400 μm palmitic acid (PA) (Sigma-Aldrich, MA, USA) in 1% (w/v) serum medium with low glucose (1 g/l) for 24 hours with and without insulin stimulation (20 nM, 10 min). 10%(w/v) fatty acid-free bovine serum albumin was used to prepare PA. These pre-adipocytes and adipocytes were processed for RNA and protein analyses or glucose uptake assay.

4.4 Oil Red O Staining (publication I)

3T3-L1 pre-adipocyte cells and differentiated cells with and without MaCM were stained for lipid droplet accumulation. Cells were rinsed with PBS and fixed with 10% formalin for 1 h at room temperature. These cells were then washed again with PBS followed by incubation with 60% isopropanol for 5 min. Lastly, cells were incubated

with 0.3% Oil Red O solution for 20 min. Coverslips were then washed with distilled water and mounted with mounting media onto a slide. A panoramic digital slide scanner was used to capture images. Untreated cells were used as control.

4.5 Quantitative real-time PCR (publication I, III, IV)

Total RNA was extracted from SAT, PBMC, and cell lines using RNeasy Lipid Tissue MiniKit (Qiagen, CA, USA) and TRIZOL reagent, respectively. RNA was quantified using an Epoch spectrophotometer (BioTek, VT, USA), and High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used for cDNA synthesis. qRT-PCR was performed using the Applied Biosystem7500 or Rotor-Gene Q system with Taqman gene expression assays or custom primers normalized to GAPDH, respectively. The primers used are summarized in Table 2. Gene expression was assessed using the ΔΔCT method and reported as fold changes.

Table 2: List of primers used for RT-PCR analysis

Gene	***Catalogue # or sequence***
Human Ucn3	Hs00846499 *(Applied Biosystem, USA)*
Human GAPDH	Hs02786624 *(Applied Biosystem, USA)*
Human UCN1	*Hs01849155 (Applied Biosystem, USA)*
Human UCN2	*Hs00264218 (Applied Biosystem, USA)*
Human UCN3	*Hs008464991(Applied Biosystem, USA)*
Human CRF	*Hs01921237 (Applied Biosystem, USA)*
Human Spexin	*Hs00228976 (Applied Biosystem, USA)*
Human TNF-α	*Hs01113624(Applied Biosystem, USA)*
Human IL-10	*Hs00961622(Applied Biosystem, USA)*
Human IL6	*Hs00985639(Applied Biosystem, USA)*
Human CCL-2	*Hs00234140(Applied Biosystem, USA)*
Human ATF-6	*5'-ACCCACTAAAGGCCAGACG-3'* *5'-CCACGTGATTAGGGAGCTGT-3'*
Human PERK	*5'-ATGATCATTCCTTCCCTGGAT-3'* *5'-AGTCAGAGATTTTCCTCCAACC-3'*
Human IRE-1	*5'-GCAAGAGGACAGGCTCAATC-3'* *5'-ACGTCCTTTGAGCAGAATGC-3'*
Human PKR	*5'-TCGCTGGTATCACTCGTCTG-3'* *5'-GATTCTGAAGACCGCCAGAG-3'*
Human *CHOP*	*5'-TGA GGA GAG AGT TAG GTA ATT CC-3'* *5'-TTT AAA ACA GGT CAT TCC TCT GC-3'*
Mouse Ucn3	Mm00453206 *(Applied Biosystem, USA)*
Mouse GAPDH	Mm99999915 *(Applied Biosystem, USA)*
Mouse *PPARG*	Mm00*440940 (Applied Biosystem, USA)*

Mouse *CRHR2*	Mm00*438308 (Applied Biosystem, USA)*
Mouse *FABP4*	Mm00*445878 (Applied Biosystem, USA)*
Mouse *FASN*	Mm00*662319 (Applied Biosystem, USA)*
Mouse *LPL*	Mm00*434764 (Applied Biosystem, USA)*
Mouse *LIPE*	Mm00*495359 (Applied Biosystem, USA)*
Mouse *TNF*	Mm00*443258 (Applied Biosystem, USA)*
Mouse *IL6*	Mm00*446190 (Applied Biosystem, USA)*
Mouse C/EBP	*5′-GAACAGCAACGAGTACCGGGTA-3′* *5′-GCCATGGCCTTGACCAAGGAG-3′*
Mouse ACC	*5′-GAACAGCAACGAGTACCGGGTA-3′* *5′-GCCATGGCCTTGACCAAGGAG-3′*
Mouse HSP90	*5'-GGCATCGATGAAGATGAGG-3'* *5'-ACATGAGCAGAGAGCCAGGT-3'*
Mouse HSP72	*5'-TGCTGATCCAGGTGTACGAG-3′* *5'-CGTTGGTGATGGTGATCTTG-3′*
Mouse HSP60	*5'-CGTTGCCAATAACACAAACG-3'* *5'-CTTCAGGGGTTGTCACAGGT-3'*
Mouse GRP78	*5'-GGATGCGGACATTGAAGACT-3′* *5'-TCCCAACGAAAGTTCCTGAG-3′*
Mouse CHOP	*5'-CGGAACCTGAGGAGAGAGTG-3′* *5'-TATAGGTGCCCCCAATTTCA-3'*
Mouse ATF6	*5'-GGTTCAGCCCTGGACAAATA-3'* *5'-CTGCTGATTAGCCGAGTTCC-3'*
Mouse PERK	*5'-GCCCAAACATCGAGAAAATG-3'* *5'-GCTCCCAGCTTCTGCTTAGA-3′*
Mouse IRE1	*5'-GGCCACTTTGAACTTCGGTA-3'* *5'-CCAATCAGCAACGGAAACTT-3'*

4.6 Western blot analysis (publication I, III, IV)

Whole-cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentration was quantified by the Bradford method. 20 µg of protein was prepared (for loading) using sample loading buffer containing β-mercaptoethanol heated at 95˚C for 10 min. The samples were then resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes at 100 V for 75 min, followed by membranes blocking with 5% non-fat dried milk for 2 h at RT. Membranes were subsequently incubated with primary antibodies at 4°C, overnight. Primary antibodies used were: anti-UCN3 (bs-2786R, Bioss Antibodies Inc., MA, USA), anti-HSP90 (ADI-SPA-830-F, Enzo), anti-HSP72 (ADI-SPA-810-F, Enzo), anti-HSP60 (ADI-SPA-805-F, Enzo), anti-GRP78 (ab32618, Abcam), anti-CHOP (2895S, Cell Signaling Technology, Danvers, MA, USA), p-AKT-ser 473/AKT, p-JNK/JNK, p-ERK/ERK,

(9271, 9272, 4668s, CST 9252L, 9101, and 9102, respectively; Cell signaling) and anti-GAPDH (ab2302, Millipore, Temecula, CA)]. Membranes were then washed and incubated with secondary antibody conjugated with horse-radish peroxidase for 2h at RT. Finally, the detection was performed using super sensitivity west Femto ECL reagent (Thermo Scientific, USA), and protein bands were visualized by chemiluminescence. Images were captured using Versadoc 5000 system (Bio-Rad, USA), and band intensities were quantified using Quantity One software (Bio-Rad, USA). GAPDH was used as the internal protein loading control.

4.7 Glucose (2-NBDG) uptake assay (publication IV)

3T3-L1 pre-adipocytes and adipocytes were plated onto 96 well plates for glucose uptake cell-based assay (#600470, Cayman Chemical, Ann Arbor, MI, USA). Following transfections and treatments, cells were starved using a glucose-free medium for 1h. The medium was then replaced with fluorescently labeled Deoxy-D-glucose analog (2-NBDG) (150 µg/mL), with and without insulin (0.1 µM) at 37 °C for 1 h. Following treatment, cells were washed, and fluorescence was measured using a Synergy H4 plate reader (BioTek, Winooski, VT, USA). Treatments and measurements were done using transfected pre-adipocytes differentiated up to 3rd day.

4.8 Statistical analysis

All statistical analyses were done using SPSS v27.0 software (v27.0; SPSS Inc., IL, USA), and at least 3 repeats were done per experimental condition. p-values < 0.05 were considered statistically significant.

Descriptive statistics have been reported as mean ± standard deviation. Categorical and skewed variables were tested using the Chi-square test and Wilcoxon non-parametric t-test. Differences between pairs of cellular experiments have been assessed using Mann-Whitney non-parametric U tests. Effects of groups in the AHS cohort were evaluated using two-way ANOVA with Bonferroni post hoc test on the whole population. Differences in groups' mean prior to and following exercise were

evaluated using paired t-test. Differences in groups' mean prior to and following exercise were evaluated using paired t-test. Correlation analysis between variables was performed using Spearman's correlation coefficient. Multiple linear regression analysis with odds ratios (ORs) was done to examine the predictive effect of the variables with a 95% confidence interval calculated for each associated factor.

For the COP cohort, categorical variables were calculated as numbers and percentages using frequency statistics. Normalization using SPSS log transformation was done for the neuropeptide variables. One-way ANOVA with Bonferroni posthoc correction for multiple comparisons was used to calculate the effects of groups on the whole population. Correlation analysis was done using partial correlation and Spearman's rank correlation for the whole study cohort and sex-specific analysis. Multivariate stepwise linear regression analysis was used to evaluate the predictive effect of the chosen variables with and without adjustments for age and sex.

For the cellular experiments with four conditions, differences in means for the pairs of experimental cellular conditions were evaluated using one-way repeated measures ANOVA with Sidak´s post hoc test. Data from three experiments were considered, and a p-value <0.05 was considered significant. Differences in means for the pairs of experimental cellular conditions were evaluated using Mann-Whitney non-parametric U tests. Spearman rank correlation analysis was performed to evaluate the associations among variables.

5 Results

5.1 Characteristics of the study cohorts

The characteristics of the Adult human study (AHS) from publication I and the Childhood obesity program (COP) study from publications II and III have been summarized (Table 1 in the publications I, II, and III, respectively.)

The AHS cohort included 3 groups: normal-weight, overweight without, and overweight with T2D (Table 3). The lipid profile, as reflected by lower HDL cholesterol ($p<0.01$) and higher triglyceride (TGL) ($p< 0.001$) concentrations, was dysregulated more in the overweight group with T2D compared with the normal-weight and overweight people without T2D. The non-diabetic overweight group had a significantly lower maximal oxygen consumption rate (VO_{2max}, $p<0.001$) and higher systolic blood pressure (SBP) ($p<0.01$) than normal-weight people. The diabetic, overweight group had higher SBP ($p<0.05$) and lower VO_{2max} ($p<0.01$) compared to overweight people without diabetes. Significantly higher concentrations of leptin and gastric inhibitory polypeptide (GIP) were observed in the overweight non-diabetic group than the normal-weight group ($p<0.05$). Glucagon and glucagon-like peptide (GLP-1) concentrations were higher in the overweight group with T2D compared with the overweight non-diabetic group ($p<0.05$). Plasma UCN3 levels were markedly lower in overweight non-diabetic people ($p<0.001$) than normal-weight participants. However, plasma UCN3 concentrations were significantly higher in the overweight adult cohort with diabetes ($p<0.01$) compared to the overweight non-diabetic group.

Table 3: Physical, clinical, and biochemical characteristics of the AHS group at baseline.

	Normal weight (n=37)	**Overweight non-diabetic (n=107)**	**Overweight diabetic (n=98)**	***Sig.***
Physical and clinical characteristics				
Gender (M/F)	14/23	42/65	53/45	0.379
Age (years)	40 ± 11	42 ± 12	52 ± 9$^{\$\$\$}$	**<0.0001**
BMI (kg/m^2)	22.3 ± 1.9	31.9 ± 4.5 ***	32.3 ± 3.7	**<0.0001**
PBF (%)	29.5 ± 5.7	36.6 ± 5.9 ***	36.1 ± 5.4	**<0.0001**
Waist (cm)	77.1 ± 9.6	99.7 ± 12.2 ***	105.7 ± 10.5$^{\$\$}$	**<0.0001**

Hip (cm)	98.0 ± 5.6	113.7 ± 11.9 ***	111.4 ± 11.6	**<0.0001**
SBP (mmHg)	106 ± 10.1	115 ± 10.6 **	119.1 ± 11.7$	**<0.0001**
DBP (mmHg)	71 ± 6.6	74 ± 7.3	75.6 ± 6.3	0.091
WBC10	6.2 ± 1.6	6.4 ± 1.6	7.5 ± 2.0$$$	**<0.0001**
VO_{2max} (ml/kg/min)	23.0 ± 5.1	18.0 ± 3.8 ***	15.7 ± 4.2$$	**<0.0001**
Metabolic markers				
Cholesterol (mmol/l)	5.06 ± 1.07	5.10 ± 0.91	4.84 ± 1.33	0.248
HDL (mmol/l)	1.46 ± 0.43	1.31 ± 0.38	1.14 ± 0.37$$	**0.0003**
LDL (mmol/l)	3.11 ± 0.88	3.27 ± 0.85	2.97 ± 1.17	0.102
TGL (mmol/l)	0.89 ± 0.78	1.15 ± 0.63	1.64 ± 1.16$$$	**<0.0001**
FPG (mmol/l)	5.12 ± 0.66	5.34 ± 0.85	8.37 ± 3.12$$$	**<0.0001**
HbA_{1c} (%)	5.5 ± 0.4	5.7 ± 0.9	7.8 ± 1.8$$$	**<0.0001**
Insulin (ng/ml)	2.74 ± 1.15 (n=37)	3.68 ± 2.19* (n=39)	3.98 ± 1.95 (n=62)	**0.012**
HOMA-IR	0.60 ± 0.24	0.89 ± 0.37*	-	**<0.0001**
Hormonal markers				
Ghrelin (pg/ml)	920 ± 636	564 ± 225***	550 ± 232	**<0.0001**
GIP (pg/ml)	431 ± 267	770 ± 684*	888 ± 585	**0.005**
GLP-1 (pg/ml)	255 ± 45	272 ± 50	295 ± 64$	**0.004**
Glucagon (pg/ml)	145 ± 46	159 ± 45	179 ± 55$	**0.006**
Leptin (ng/ml)	4.4 ± 2.6	9.2 ± 5.6***	7.9 ± 4.6	**<0.0001**
Inflammatory markers				
IL-6 (pg/ml)	17.3 ± 4.8	17.1 ± 5.8	18.9 ± 8.7	0.2931
TNF-**α** (pg/ml)	138 ± 32	128 ± 38	140 ± 81	0.4590
RANTES (ng/ml)	7.5 ± 1.3	7.9± 1.6	8.2±1.4	0.159
PAI-1 (ng/ml)	13.1 ± 5.2	15.2 ± 4.9	17.0 ± 6.4	**0.007**
Other markers				
UCN3 (ng/ml)	11.99(0.78-86.07)	6.27(0.64 -77.04) ***	9.03(0.77-104.92) $$	**0.00015**

*Data are presented as the mean ± SD. * p<0.05, **p<0.01, ***p<0.001 where * is significance between normalweight and overweight non-diabetic. $ p<0.05, $$ p<0.01, $$$p<0.001, where $ is significance between overweight non-diabetic and diabetic*

The COP study population included 120 children with a mean age of 12 years and was categorized into normal-weight, overweight, and obese (Table 4).

Dysregulated lipid profile was observed in overweight and obese children compared to normal-weight children, mirrored by elevated LDL and TGL levels ($p<0.05$), The overweight and the obese group also had significantly higher glycemic markers: insulin and glucose concentrations and HOMA-IR values were elevated ($p<0.05$). Further, obese and overweight children had higher inflammatory marker TNFα and obesity markers leptin, NGAL, sICAM-1, and ZAG ($p<0.05$). While

glucagon levels were markedly lower in the overweight group, both glucagon and adiponectin levels were lower in the obese group ($p<0.05$). The children with obesity were further categorized based on HOMA-IR into metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO), wherever MHO had HOMA-IR <3.16 and MUO had HOMA-IR ≥3.16 (Table S1 in publication II). As expected, BMI and body fat percentage were significantly higher in the MUO group when compared to the MHO group ($p<0.001$).

Table 4: Physical, clinical, and biochemical characteristics of the COP group.

	Mean ± SD			**p-value**			**Overall p-value**
	Normal weight (NW) (n = 21)	**Overweight (OW) (n = 14)**	**Obese (OB) (n = 85)**	**NW vs OW**	**NW vs OB**	**OW vs OB**	**All children**
Physical and clinical characteristics							
Sex (M/F)	13/8	4/10	50/35	0.829	0.054	0.032	0.085
Age (years)	11.9 ±4.0	13.1 ±3.0	13.0 ±2.9	0.307	0.125	0.954	0.288
Weight (kg)	45.4 ±19.1	61.7 ±13.4	87.9 ±26.5	**0.009**	**<0.001**	**<0.001**	**<0.001**
Height (m)	1.50 ±0.21	1.57 ±0.12	1.60 ±0.15	0.311	**0.024**	0.524	0.065
BMI (kg/m^2)	18.9 ±3.5	24.7 ±2.8	33.8 ±6.4	**<0.0001**	**<0.0001**	**<0.0001**	**<0.001**
Percentile	54.9 ±19.8	92.0 ±1.6	98.5±1.1 #	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Body fat (%)	7.3 ±6.0	18.4 ±6.7	32.8 ±13.0	**<0.0001**	**<0.001**	**<0.001**	**<0.001**
Metabolic and Hormonal markers							
Cholesterol (mmol/l)	3.9 ±0.5	4.3 ±0.6	4.3 ±0.7	0.113	0.060	0.966	0.150
HDL (mmol/l)	1.48 ±0.27	1.35 ±0.24	1.20 ±0.45	0.145	**0.001**	0.265	**0.017**
LDL (mmol/l)	2.20 ±0.49	2.60 ±0.61	2.65 ±0.72	**0.043**	**0.008**	0.798	**0.026**
TGL (mmol/l)	0.65 ±0.30	0.92 ±0.45	1.00 ±0.43	**0.037**	**<0.001**	0.553	**0.003**
GLU (mmol/l)	4.8 ±0.4	5.4 ±1.7	5.1 ±0.4	**0.048**	**0.025**	0.439	**0.046**
Total Bilirubin (mmol/l)	10.4 ±5.8	10.8 ±3.9	8.1 ±2.9	1.000	0.078	0.086	**0.017**
ALT (mmol/l)	26.1 ±6.8	23.1 ±6.6	37.2 ±22.3	0.245	**0.036**	**<0.001**	**0.014**
HbA_{1c} (%)	5.2 ±0.3	5.56 ±1.7	5.62 ±2.3	0.443	0.141	0.932	0.712
Insulin (mIU/l)	5.5 ±3.0	9.3 ±3.9	18.2 ±13.7	**0.003**	**<0.001**	**0.024**	**<0.001**
HOMA-IR	1.23 ±0.74	1.95 ±0.75	4.32 ±3.42	**0.016**	**<0.001**	**0.014**	**<0.001**
Adiponectin (µg/ml)	9.8 ±1.4	8.9 ±1.4	8.1 ±2.0	0.065	**<0.001**	0.153	**0.001**
TNF-α (ng/ml)	4.3 ± 12.8	32.8 ±8.8	35.0 ± 33.0	**0.014**	**<0.001**	1.000	**<0.001**
Other markers							
Leptin (ng/ml)	7.5±2.6	13.3±7.0	16.1±7.5	**0.002**	**<0.001**	0.190	**<0.001**
Glucagon (ng/ml)	3.59 ±1.53	1.92 ±1.20	2.35 ±0.98	**0.002**	**<0.001**	0.147	**<0.001**
NGAL (ng/ml)	22.9 ±6.1	35.7 ±15.1	33.5 ±13.9	**0.009**	**0.001**	0.589	**0.004**
RBP4 (µg/ml)	20.2 ±5.5	55.30 ±25.1	63.76 ±103.2	**<0.001**	0.064	0.770	0.147
sICAM-1 (ng/ml)	128.3 ±78.8	255.2 ±199.4	217.3 ±252.2	**0.015**	**0.019**	0.611	0.204

ZAG (µg/ml)	1.6 ±0.2	4.9 ±0.6	3.3 ±2.0	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Resistin (ng/ml)	4.9 ±2.7	4.6 ±1.4	5.4 ±2.2	0.706	0.507	0.280	0.487

#: Only percentile between 95 and 99 were included for the obese children.Data are presented as the mean ± SD

A subset of the COP study cohort was included in the PBMC transcriptomics study (publication III). This subset study cohort consisted of 40 children with a mean age of 12 years stratified into normal weight, overweight, and obese. Their anthropometric, clinical, and metabolic characteristics are summarized in table 5.

Overweight and obese children had significantly higher body fat-%, BMI, and impaired lipid profile than normal-weight children ($p<0.05$). Insulin and HOMA-IR were also significantly higher in overweight and obese children ($p<0.05$) when compared to normal-weight children. The obesity markers leptin, NGAL, RBP4, sICAM-1, and ZAG were elevated in overweight compared to normal-weight children. As expected, inflammation, as reflected by TNFα, was markedly elevated with BMI compared to normal-weight participants. Children with obesity had significantly higher body fat percentile, insulin, and HOMA-IR levels than normal-weight children. Impaired lipid profiles with evidently lower HDL levels were observed in children with obesity. Circulating UCN2 and UCN3 levels were markedly higher in the overweight group ($p<0.05$). C-peptide and glucagon were significantly lower in the obese group. Compared to the overweight and obese groups, circulating CRF, UCN2, and UCN3 were lower in the obese groups; however, Spexin levels were elevated.

Table 5: Physical, clinical, and biochemical characteristics of the COP group in the PBMC transcriptomics study

	Mean ± SD			**p-value**			**Overall p**
	Normal weight, NW (n = 8)	***Overweight, OW (n = 10)***	**Obese (OB) (n = 22)**	***NW vs OW***	***NW vs OB***	***OW vs OB***	**All children**
Physical and clinical characteristics							
Sex (M/F)	5/3	3/7	14/8	0.188	0.956	0.082	0.196
Age (years)	10.5 ± 4.3	12.8 ±3.4	11.3±2.1	0.260	0.788	0.429	0.265
Weight (Kg)	38.2 ± 18.1	58.5±14.6	83.6±30.9	0.230	**<0.001**	**0.037**	**<0.001**
Height (m)	1.44 ± 0.25	1.54±0.12	1.52±0.17	0.482	0.511	0.969	0.460
BMI (Kg/m^2)	17.16±2.59	24.22±2.97	35.05±6.95	**0.029**	**<0.001**	**<0.001**	**<0.001**
Percentile	41.0 ± 18.8	91.8±1.8	98.8±0.6	**<0.001**	**<0.001**	0.078	**<0.001**
Body fat (%)	6.1 ±3.6	16.7±6.8	32.9±15.3	**0.003**	**<0.001**	**0.005**	**<0.001**

Metabolic and Hormonal markers							
Cholesterol (mmol/l)	4.09 ±0.62	4.57±0.51	4.11±0.96	0.451	0.997	0.357	0.344
HDL (mmol/l)	1.43 ±0.31	1.34±0.15	1.10±0.28	0.748	**0.013**	0.074	**0.008**
LDL (mmol/l)	2.33 ±0.59	2.82±0.44	2.57±0.82	0.323	0.692	0.639	0.355
TGL (mmol/l)	0.71 ±0.36	0.99±0.51	1.00±0.45	0.410	0.295	0.999	0.297
GLU (mmol/l)	4.5 ±0.4	5.6±2.0	5.0±0.4	0.120	0.633	0.326	0.132
HbA_{1c} (%)	5.2 ±0.3	5.7±2.0	5.2±0.7	0.670	0.998	0.525	0.528
Insulin (mIU/L)	3.6 ±1.8	9.4±4.4	17.15±6.8	**0.003**	**0.016**	0.08	**0.012**
HOMA IR	0.74 ±0.39	2.59±2.34	5.54±5.43	**0.043**	**0.001**	**0.010**	**0.001**
Adiponectin (µg/ml)	9.8 ± 1.1	8.6±1.3	7.6±2.7	0.496	0.057	0.473	0.065
TNFα (ng/ml)	1.21 ± 0.59	31.79±9.40	25.86±34.60	**0.046**	0.073	0.826	0.344
Other markers							
Leptin (ng/ml)	7.78 ± 2.88	13.12±6.67	11.17±4.04	0.053	0.197	0.521	**0.041**
C-pep (ng/ml)	366 ±219	187±75	112±41	**0.006**	**<0.001**	0.221	0.064
Glucagon (ng/ml)	4.04 ± 1.81	1.62±0.51	2.38±1.14	**<0.001**	**0.005**	0.236	**<0.001**
NGAL (ng/ml)	24.71 ±8.59	36.57±17.87	24.31±5.02	0.058	0.996	**0.013**	**<0.001**
RBP4 (µg/ml)	19.45 ± 2.92	54.97±19.74	19.10±5.75	**<0.001**	0.997	**<0.001**	**0.013**
sICAM-1 (ng/ml)	132 ± 31	284±233	157±83	0.057	0.890	0.057	**<0.001**
ZAG (µg/ml)	1.60 ± 0.22	5.02±0.71	1.70±0.37	**<0.001**	0.847	**<0.001**	**0.037**
Resistin (ng/ml)	5.16 ± 2.98	4.76± 1.46	6.75±2.92	0.950	0.337	0.166	**<0.001**
Circulating CRF (pg/ml)	76.80 ±103.89	136.34±105.2	23.38±44.55	0.242	0.222	**0.001**	0.132
Circulating UCN1 (pg/ml)	43.38 ±42.12	15.35±27.67	32.30±32.41	0.195	0.704	0.388	**0.002**
Circulating UCN2 (ng/ml)	1297 ±362	1832±40	1397±374	**0.014**	0.800	**0.013**	0.206
Circulating UCN3 (ng/ml)	2.94 ±0.99	33.28±37.81	2.33±0.32	**0.006**	0.997	**0.001**	**0.007**
Circulating Spexin (ng/ml)	14.67 ± 1.59	13.14±2.13	18.04±4.38	0.648	0.071	**0.004**	**0.001**

#: Only percentile between 95 and 99 were included for the obese children. Data are presented as the mean ± SD.

5.2 Circulating plasma UCN3 levels are affected by obesity, T2D and sex

In the AHS study cohort, there was a significant decrease in circulating UCN3 levels with increased body mass index (BMI) in the overweight subjects compared to normal-weight participants. On the other hand, there were significantly higher plasma UCN3 levels with T2D compared to non-diabetic overweight participants ($p<0.01$) (Table 1 in publication I).

Taking into account that in the overweight group, there were higher UCN3 levels with T2D, we further stratified the diabetic group based on HbA_{1c} values into well-controlled and uncontrolled HbA_{1c} groups (HbA_{1c} < 6.5% and HbA_{1c} ≥6.5%, respectively). UCN3 levels were significantly higher in the well-controlled group than the uncontrolled HbA_{1c} group (11.7±8.8 ng/ml vs. 5.9±3.6, respectively, $p<0.05$).

In the COP study cohort, the plasma levels of UCN1-3, CRF, and Spexin neuropeptides were assessed for the whole population and stratified into groups according to BMI (Figure S1 in publication II). Circulating UCN1 levels were significantly lower in the overweight group than normal-weight children (15.6 ± 6.4 pg/ml and 37.7 ± 7.6 pg/ml, respectively, $p<0.05$). However, the UCN1 concentration was elevated with obesity (43.1 ± 9.5 pg/ml) to similar levels in the normal-weight group (Figure S1A in publication II). Plasma UCN2 levels were significantly higher in the overweight group in comparison to the normal-weight children (1789 ± 103 ng/ml and 1477 ± 101 ng/ml, respectively, $p<0.05$), however, a statistically significant increase was not seen in the obese group (Figure S1B in publication II). Plasma UCN3 levels were elevated with increasing BMI, particularly in the overweight group compared to the normal-weight group ($p < 0.001$, Figure S1C in publication II). On the other hand, no significant changes in CRF and Spexin levels were observed with BMI (Figure S1D and S1E in publication II).

To account for any potential influence of the sex on the results, we further grouped the cohort by sex and assessed the levels of neuropeptides (Figure 1 in publication II). Although UCN1 levels were generally lower in females irrespective of BMI, this decrease was statistically significant only in normal-weight girls compared to normal-weight boys (19.3 ± 25.7 pg/ml and 49.1 ± 34.8 pg/ml, respectively, $p<0.05$, Figure IA). Plasma UCN2 and UCN3 levels were higher in obese girls than boys, and plasma UNC2 concentrations were higher in overweight girls than boys (Figure 1B and 1C in publication II). All-female subgroups tended to have elevated CRF levels compared with boys. However, this increase was statistically significant only in the obese girls (Figure 1D in publication II). Spexin levels did not vary between boys and girls (Figure 1E in publication II). Further, there were no significant changes in the levels

of all the studied neuropeptides between MHO and MUO groups (Table S1 in publication II)

5.3 UCN1, UCN3 and CRF mRNA levels in circulating PBMCs are affected by obesity

Considering the dysregulation in plasma levels of UCN1-3 and CRF, we further assessed the mRNA expression levels of these neuropeptides in blood cells, particularly in the peripheral blood mononuclear cells (PBMCs) (publication III) from the three groups - normal weight, overweight and obese.

In the COP group for the PBMC transcript study, UCN1, UCN3, and CRF mRNA expression reduced significantly in overweight and obese children compared to normal-weight children (Figure 6). There was more variation in UCN2 levels within each group, and no trend could be observed between groups. Spexin mRNA expression between normal-weight, overweight or obese groups increased with increased body weight; however, it did not reach statistical significance.

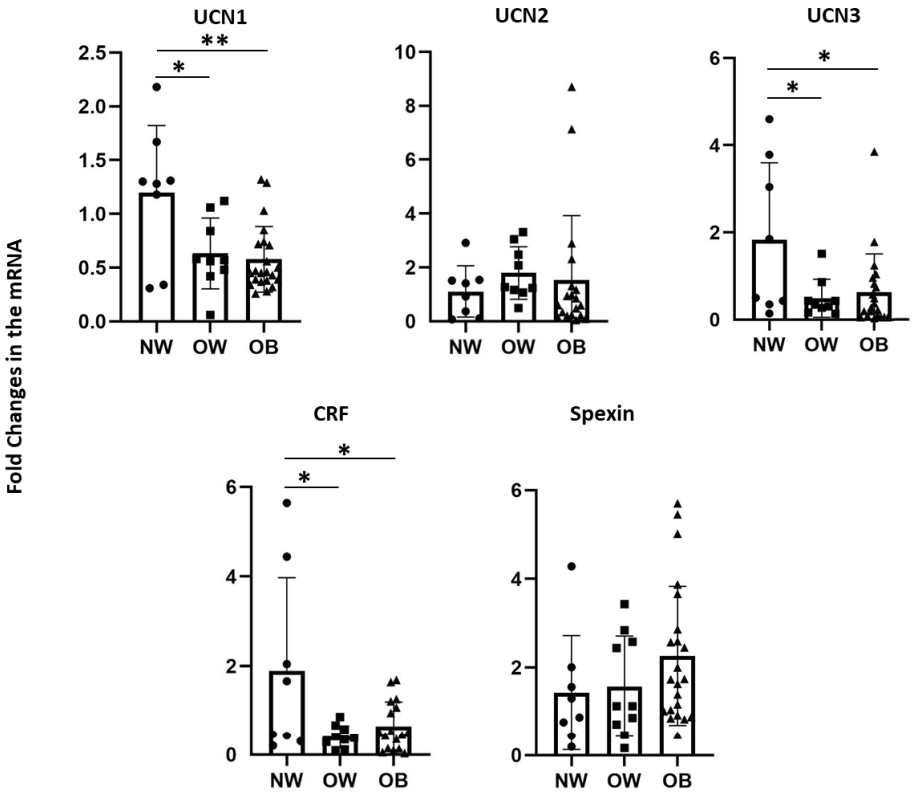

Figure 6: Urocortin 1,2,3; CRF, and Spexin mRNA expression levels in the PBMCs according to bodyweight group in children. *mRNA levels of UCN1* ***(A)****, UCN2* ***(B)****, UCN3* ***(C)****, CRF* ***(D)****, and Spexin* ***(E)****, were measured by RT-PCR using PBMC samples from normal weight (NW, n = 10), overweight (OW, n = 11), and obese children (OB, n = 25). Data are normalized to internal GAPDH and presented as fold changes compared with the normal-weight group. Data are expressed as mean±SD, * p < 0.05 and ** p < 0.01.*

5.4 Association of plasma UCN3 levels with clinical and metabolic parameters

In the AHS cohort, Spearman's correlation analysis was performed for the total study population (Table 2 in publication I) and separately for groups with normal weight or overweight with and without diabetes. Plasma UCN3 levels were directly correlated with GLP1, visfatin, and TGL ($p<0.05$) in the total population and diabetic and non-diabetic overweight groups. Plasma UCN3 levels were directly correlated

with glycemic markers FPG and HbA_{1C}, SBP, and glucagon but were inversely correlated with insulin, RANTES, and leptin ($p<0.05$). In the non-diabetic overweight participants, there was a significant direct correlation between UCN3 levels and HbA_{1c}, and in the diabetic, overweight adults, there was a significant inverse association with RANTES ($p<0.05$).

Multivariable linear regression analysis was then performed to study the independent association of UCN3 with these metabolic markers (Table 3 in publication I). In the total study cohort and diabetic, overweight patients, independent associations were observed between UCN3 and RANTES, HbA_{1c}, fasting plasma glucose (FPG), and GLP1 ($p<0.05$). In the non-diabetic overweight adults, UCN3 levels were independently associated with HbA_{1c} ($p<0.05$). Furthermore, odds ratio analysis for overweight groups with normal-weight groups as reference (Table 4 and Table S3 in publication I) showed a strong association between UCN3 and adiposity parameters in diabetic and non-diabetic overweight participants.

Association studies in the COP cohort

In the COP cohort, a partial correlation analysis, adjusted for age and sex, was performed to study the association of circulating neuropeptides UCN1-3, CRF, and Spexin with various metabolic markers in Table 4 (publication III).

UCN1 correlated directly with total serum protein, albumin, and height. UCN3 was directly associated with the inflammatory marker TNFα, obesity markers RBP4 and ZAG, and body fat percentage and inversely associated with C-peptide. UCN2 was directly associated with CRF, while CRF only correlated inversely with AST.

Association studies were done separately for girls and boys (Table 3 in publication II). UCN1 was inversely associated with albumin only in boys. UCN1 correlated inversely with ZAG, TNFα, and HDL in girls. UCN2 was directly associated with C-peptide and glucagon and inversely with alkaline phosphatase in boys. In girls, UCN2 correlated inversely with leptin. UCN3 correlated inversely with glucagon in girls and directly with TNFα, leptin, RBP4, and ZAG in boys. CRF was inversely associated with serum albumin, and Spexin was directly correlated with AST in girls.

Multivariable stepwise linear regression analysis was performed in the whole study cohort after adjustment for age and sex, with each neuropeptide marker as a dependent variable (Table 4 in publication II). In contrast, UCN1 was independently

associated with TNFα and UCN3 ($p<0.05$), UCN2 was associated with UCN3 and CRF. ZAG and UCN2 were independently associated with UCN3 ($p<0.05$), and CRF was independently associated with UCN2 and Spexin ($p<0.05$).

5.5 Association of UCN3 PBMC transcript levels with clinical and metabolic parameters

Correlation analysis was performed to study the association between fold change levels of mRNA of each neuropeptide and metabolic parameters as shown in Table 6 (Table 2 in publication III).

Significant inverse associations were observed between UCN1 expression, obesity markers (BMI, percentile, and body fat percentage), and serum cholesterol. UCN3 negatively correlated with TNFα while CRF was inversely associated with TNFα, RBP4, ZAG and circulating levels of UCN2. UCN2 and UCN3 transcript levels were associated directly with circulating UCN3 levels, and expression levels of CRF UCN1-3 correlated directly with each other.

Table 6: Spearman correlation ranking of Urocortin PBMC transcript fold change (FC) with characteristics of the participants.

All children	FC CRF	FC UCN1	FC UCN2	FC UCN3	FC Spexin
Sex (M/F)	-0.045	-0.023	0.063	-0.089	-0.039
Age (years)	-0.323	-0.169	-0.186	-0.069	-0.186
Weight (kg)	-0.091	**-0.352***	0.026	-0.129	0.093
Height (m)	-0.030	-0.240	-0.030	-0.033	-0.085
BMI (kg/m^2)	-0.141	**-0.362***	-0.295	-0.308	0.291
Percentiles	-0.132	**-0.351***	-0.143	-0.234	0.196
Body fat (%)	-0.019	**-0.423***	0.087	-0.051	0.059
Cholesterol (mmol/l)	-0.101	**-0.427****	0.031	-0.164	0.120
HDL (mmol/l)	0.145	0.071	0.011	0.094	0.125
LDL (mmol/l)	0.058	0.261	-0.130	0.140	-0.232
TGL (mmol/l)	0.144	-0.034	0.058	0.037	0.252
Fasting glucose(mmol/l)	-0.043	0.047	0.127	-0.039	0.208
HbA_{1C} (%)	-0.203	-0.039	0.028	-0.230	-0.032
Insulin (mIU/L)	-0.040	-0.047	0.182	0.075	-0.244
HOMA IR	-0.239	-0.333	-0.256	-0.317	0.048
Adiponectin (µg/ml)	0.086	0.254	-0.139	0.003	-0.019
TNFα (ng/ml)	**-0.387***	-0.050	-0.208	**-0.388***	0.083
Leptin (ng/ml)	-0.197	-0.005	0.176	-0.212	0.289

C-pep (pg/ml)	0.069	0.257	0.193	0.219	-0.121
glucagon (ng/ml)	0.269	0.119	-0.025	0.221	-0.081
NGAL (ng/ml)	-0.220	0.256	0.066	**-0.343***	0.206
RBP4 (µg/ml)	**-0.415***	-0.027	-0.056	-0.328	-0.175
sICAM-1 (ng/ml)	-0.323	0.067	0.174	-0.122	-0.136
ZAG (µg/ml)	**-0.428***	-0.082	0.054	-0.224	-0.190
Resistin (ng/ml)	-0.068	-0.235	-0.078	-0.205	0.057
Circulating CRF (pg/ml)	-0.327	0.091	0.058	-0.081	-0.228
Circulating UCN1 (pg/ml)	0.097	-0.209	-0.121	-0.090	0.114
Circulating UCN2 (ng/ml)	**-0.353***	-0.274	-0.215	-0.209	-0.014
Circulating UCN3 (ng/ml)	0.050	0.298	**0.545****	**0.331***	-0.083
Circulating Spexin (ng/ml)	0.106	-0.304	**-0.439***	-0.263	0.307
FC CRF	1.000	0.293	**0.663****	**0.668****	0.258
FC UCN1	0.293	1.000	0.377	**0.409***	0.071
FC UCN2	**0.663****	0.377	1.000	**0.887****	0.163
FC UCN3	**0.668****	**0.409***	**0.887****	1.000	-0.056
FC SPX	0.258	0.071	0.163	-0.056	1.000

*Data are presented as the R-values. * $p<0.05$, ** $p<0.01$.*

5.6 UCN3 expression is augmented in SAT from overweight adult individuals and decreased by physical exercise and inflammation

Considering the dysregulation of plasma UCN3 levels with obesity and diabetes, we assessed UCN3 expression in subcutaneous adipose tissue (SAT) (publication I). Representative SAT samples were obtained from the three groups of the AHS cohort: normal-weight, non-diabetic overweight, and overweight with T2D. UCN3 expression showed a different pattern in SAT compared to plasma UCN3. A significant elevation in UCN3 expression was observed in non-diabetic overweight participants compared to normal-weight individuals at both mRNA and protein levels ($p<0.001$ and $p<0.01$, respectively) (Figure 1A and 1B in publication I). Conversely, a significant reduction in UCN3 expression was observed in diabetic, overweight individuals compared to non-diabetic overweight people at mRNA and protein levels ($p<0.001$and $p<0.05$, respectively) (Figure 1A and 1B in publication I). Staining for adiponectin was also done as a control for SAT from the same representative SAT samples. Non-diabetic overweight people had lower adiponectin levels than normal-weight people with a further decrease in diabetic, overweight participants (Figure S1B in publication I).

Our previous studies have reported differential expression patterns of stress and inflammatory pathways in SAT from normal-weight and obese people with or without T2D (Khadir, Kavalakatt, et al. 2018a; Khadir, Kavalakatt, Madhu, et al. 2018). In

addition, we have observed a strong correlation between plasma UCN3 and RANTES levels in the AHS study, which led us to investigate the effect of inflammation on UCN3 levels. We cultured and differentiated pre-adipocyte cells without or in the presence of macrophage conditioned media (MaCM). UCN3 is expressed in pre-adipocytes and adipocytes (day 0 (D0) and day 8 (D8), respectively) at both proteins (Figure 2A in publication I) and mRNA (Figure 2B in publication I) levels. Oil Red O staining of the lipid droplet accumulated was performed to assess differentiation (Figure 2 D in publication I). With differentiation and maturation of adipocytes by day 8, UCN3 expression was markedly increased (Figure 2A and 2B in publication I). On the contrary, differentiation of pre-adipocytes in the presence of MaCM led to a significantly lower UCN3 (D8+MaCM) and lipid accumulation (Figure 2A, B, and D in publication I). Differentiation of adipocytes with and without MaCM was confirmed by the observed increase in mRNA levels of lipogenic markers FABP4 and FASN, C/EBP, PPARG, lipolysis markers LPL and LIPE, and inflammatory marker IL6 (Figure 2C in publication I).

5.7 Metabolic stress affects the expression levels of UCN1, UCN2, UCN3, CRF, and Spexin in THP1 cells

The dysregulation of the studied neuropeptides with obesity in PBMC transcripts prompted us to elucidate the effect of the metabolic milieu in obesity and diabetes on these peptides (publication III). For this purpose, we used human monocyte cell line THP1 and treated it with high glucose (HG), palmitic acid (PA), and a combination of both (HG+PA).

A significant decrease in CRF gene expression was observed in response to HG or PA, whereas a decrease in UCN3 gene expression was observed for all treatments when THP1 cells were exposed acutely for 4 hours (Figures 7C and 7D). The decrease in the mRNA expression of Spexin was observed with the combination treatment (Figures 7A, 7B, and 7E). The expression of UCN1 and UCN2 did not change significantly for the short duration of treatment. However, chronic exposure to PA or HG+PA for 24 h led to a significant increase in UCN2, UCN3, and CRF mRNA levels (Figures 8B-D). The effect of HG on the mRNA expression of these peptides was marginal, with only CRF and Spexin mRNA showing a statistically significant decrease (Figure 8D and 8E).

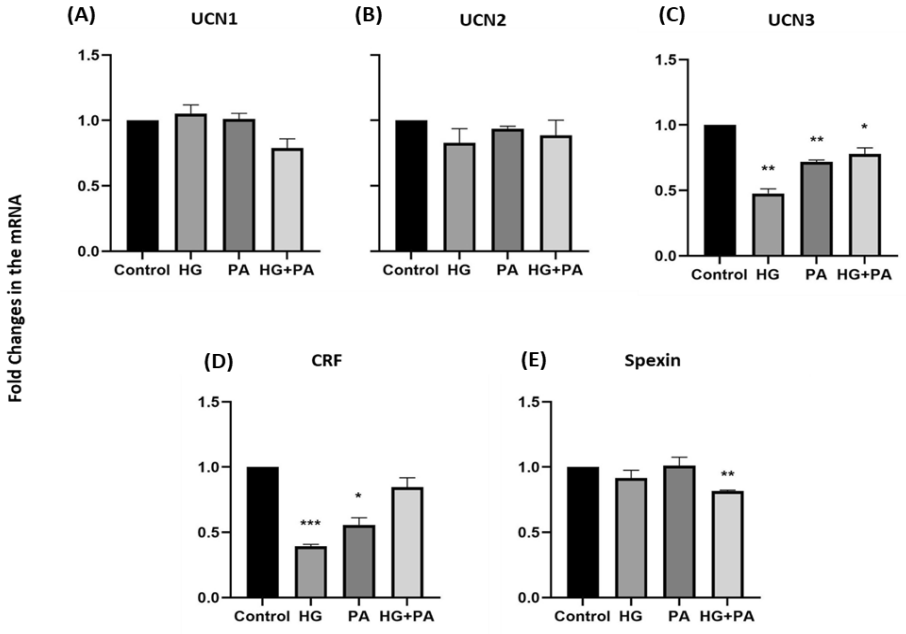

Figure 7- Gene expression levels in the THP1 cells treated for four hours. *mRNA expression levels of UCN1, UCN2, UCN3, CRF, and Spexin were measured by quantitative real-time in the THP1 cells treated with high glucose (25 mM), palmitate (200 μM) and their combination for 4 hours (n = 3 for each condition). Data are normalized to internal GAPDH and presented as fold changes compared with normal weight data. Data are expressed as mean±SD (* p < 0.05, ** p < 0.01, ***p<0.001 vs control).*

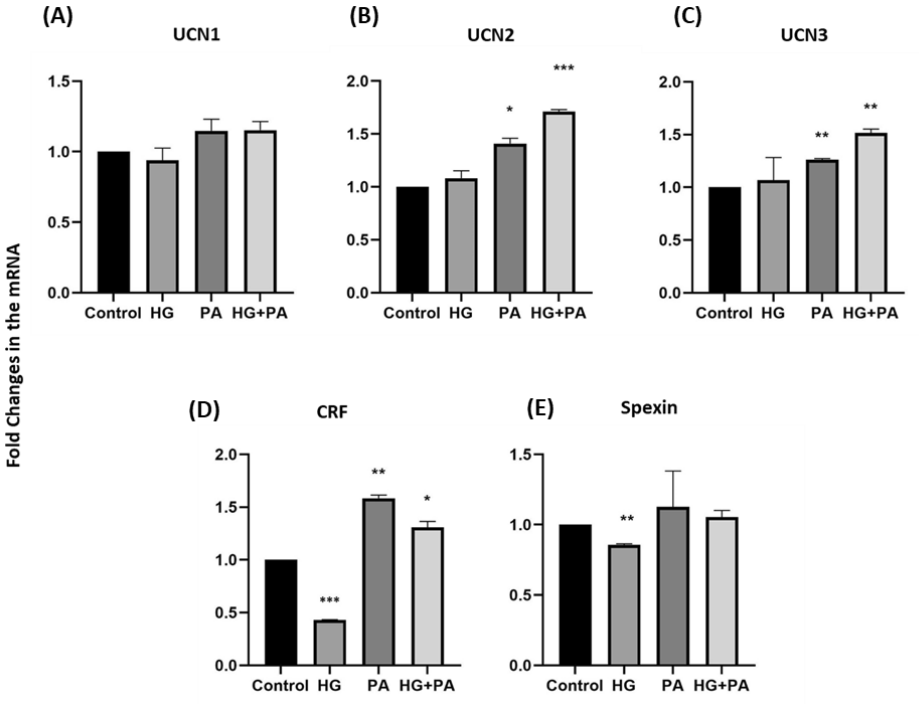

Figure 8: Gene expression levels in the THP1 cells treated for 24 hours. *mRNA expression levels of UCN1, UCN2, UCN3, CRF, and Spexin were measured by quantitative real-time in the THP1 cells treated with high glucose (25 mM), palmitate (200 µM) and their combination for 24 hours (n = 3 for each condition). Data are normalized to internal GAPDH and presented as fold changes compared to control. Data are expressed as mean ± SD (* p < 0.05, ** p<0.01, ***p<0.001 vs control).*

To further elucidate the effect of metabolic stressors on these neuropeptides, we analyzed the mRNA expression of ER stress and inflammatory markers in THP1 cells in response to treatments with HG, PA, or HG+PA for 4 h and 24 h. After 4 hours of treatment, the combination of PA and HG-induced expression of inflammatory markers (Figure 9A-D) and ER stress markers (Figure 9E-I). Further, following PA and HG+PA treatments for 24 h, a significant increase in inflammatory markers TNFα, IL10, IL6, and CCL2 (Figure 10A-D) as well as ER stress markers ATF6, PERK, IRE1, PKR, and CHOP (Figure 10E-I) was observed.

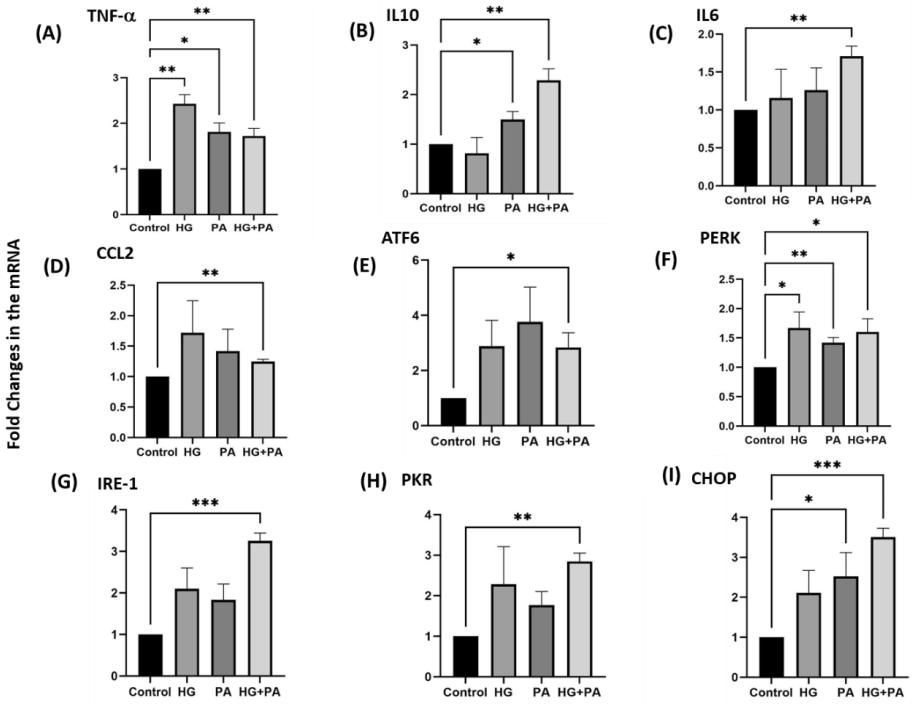

Figure 9: Gene expression levels of inflammatory and ER stress markers in THP1 cells treated for four hours. *Gene expression levels of inflammatory markers (TNFα, IL10, IL6, CCL2) and ER stress markers (ATF6, PERK, IRE1, PKR, and CHOP) were measured by quantitative real-time in the THP1 cells treated with high glucose (25 mM (HG)), palmitate (200 µM (PA)) and their combination (HG+PA) for 4 hours (n = 3 for each condition). Data are normalized to internal GAPDH and presented as fold changes compared with untreated cells (Control). Data are presented as mean ±SD (* p < 0.05, **p<0.01 and ***p<0.001).*

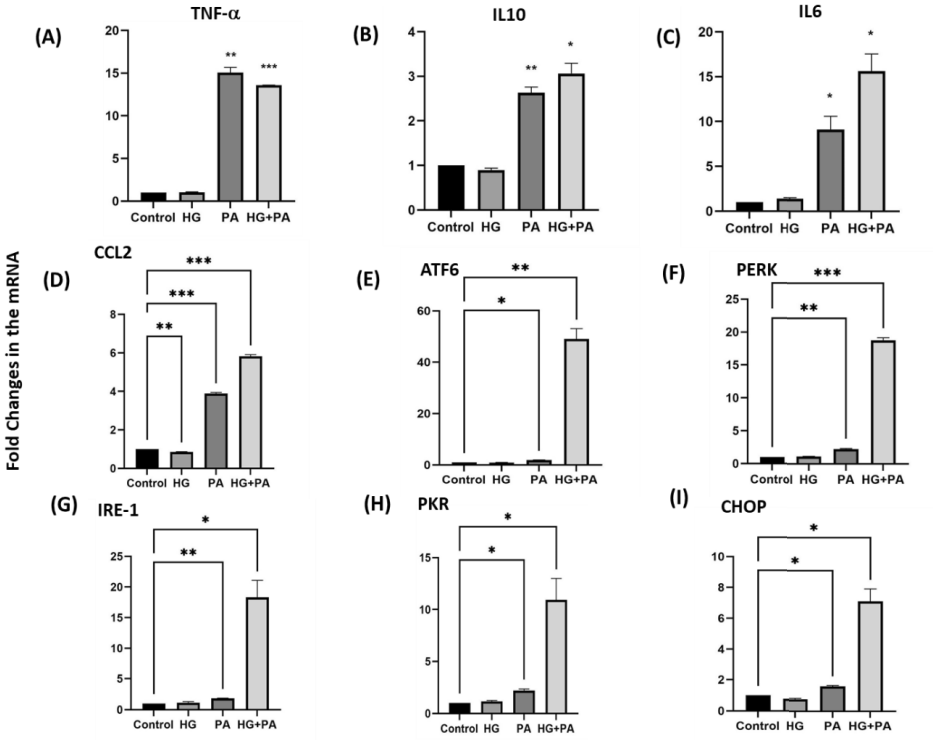

Figure 10: Gene expression levels of inflammatory and ER stress markers in THP1 cells treated for 24 hours. *Gene expression levels of inflammatory markers (TNFα, IL10, IL6, CCL2) and ER stress markers (ATF6, PERK, IRE1, PKR, and CHOP) were measured by quantitative real-time in the THP1 cells treated with high glucose (25 mM (HG)), palmitate (200 µM (PA)) and their combination (HG+PA) for 24 hours (n = 3 for each condition). Data are normalized to internal GAPDH and presented as fold changes compared with untreated cells (Control). Data are presented as mean ±SD (* p < 0.05, ** p<0.01, ***p<0.001).*

5.8 The effect of exercise on UCN3 levels in plasma and SAT

A subgroup of adult people in the AHS group underwent a supervised physical exercise regimen for three months as part of a lifestyle intervention (publication I). Our previous studies have reported the effect of exercise on various parameters, with significant changes, particularly in diabetic, overweight people (Khadir, Kavalakatt, et al. 2018a; Khadir, Kavalakatt, Madhu, et al. 2018). Considering the impairment of

UCN3 plasma and SAT levels with obesity and diabetes, we wanted to assess the effect of exercise on UCN3 levels.

Exercise effect was observed with improved VO_{2max} and significantly decreased percent body fat (PBF), BMI, and waist circumference in non-diabetic overweight people. PBF and waist circumference also decreased in overweight people with diabetes (Table S4 in publication I). Plasma UCN3 levels increased following 3 months of exercise training in non-diabetic but not diabetic, overweight people.

Exercise regimen effect was also assessed in SAT biopsies obtained from the overweight people with and without T2D. A significant decrease in SAT UCN3 expression following exercise training was observed in people with or without T2D ($p<0.01$), as shown by the representative IF images (Figure 1C in publication I).

5.9 Plasma UCN 3 is inversely associated with circulating HSP60 and GRP78 concentrations in adult humans with obesity

Based on the effects of inflammation and metabolic stressors on UCN3 levels and to further investigate the role of UCN3, we performed correlation analysis for plasma UCN3 with other stress markers in the AHS study cohort (publication IV). Interestingly, circulating UCN3 levels significantly correlated with ER stress chaperone protein- the 78 kDa- glucose-regulated protein (GRP78) and heat shock protein (HSP) 60 ($R = -0.285$, $p = 0.006$ and $R = -0.384$, $p = 0.001$, respectively) in the non-diabetic study cohort (n=144, Figure S7 in publication IV). On the other hand, there was no association in the diabetic subgroup (n=95).

5.10 UCN3 overexpression decreases inflammation

Adipose tissue from obese and insulin-resistant people is characterized by low-grade chronic inflammation (Hotamisligil 2006). Considering the dysregulation of UCN3 in obesity and diabetes in SAT and 3T3-L1 adipocytes differentiated with MaCM, we further studied the effect of MaCM on HSPs and ER stress markers (publication IV). UCN3 mRNA expression increased with adipocyte differentiation. However, when differentiated in the presence of MaCM, UCN3 mRNA levels were decreased (Figure 6 in publication IV). Adipocytes differentiated in the presence of MaCM expressed increased levels of HSP60 and GRP78 mRNA (Figure 6 in publication IV). Expression

of ER stress markers ATF6, IRE1, and PERK tended to be increased, although statistical significance was not observed (Figure 7 in publication IV). Apoptotic marker CHOP mRNA was increased in response to MaCM (Figure 6 in publication IV).

Next, we assessed the effect of UCN3 overexpression on these HSP, inflammatory, and ER stress markers. UCN3 overexpression significantly reduced apoptotic marker CHOP and ER stress markers ATF6 and IRE1 (Figure S5 in publication IV). With UCN3 overexpression, there were also significant differences in HSP90 (a decrease) and HSP72 (an increase), as shown in Figure S4 from publication IV.

5.11 UCN3 overexpression enhances cellular stress response under palmitic acid stress

Considering the possible protective effects of UCN3 and to further elucidate its role, we overexpressed UCN3 in differentiated 3T3-L1 adipocytes followed by treatment with and without 400 μM palmitic acid (PA) (Figure S1 in publication IV).

UCN3 overexpression markedly reduced the mRNA expression of HSP90, HSP72, HSP60, and GRP78 ($p<0.05$), as shown in Figure 1 in publication IV. As expected, PA treatment significantly upregulated HSP72 and HSP60 mRNA expression ($p<0.01$ and $p<0.05$, respectively), and this increase was attenuated by UCN3 overexpression. The protein expression of HSP90, GRP78, and HSP72 was significantly decreased by UCN3 overexpression in PA-treated cells (Figure 2 in publication IV).

We further studied the effect of UCN3 overexpression on ER stress markers and apoptotic marker-CHOP. Overexpression of UCN3 alone, without PA treatment, significantly reduced expression of ATF6 and IRE1 (Figure 3). The expression of CHOP (Figure 2 and 3 in publication IV) and PERK (Figure 3 in publication IV) was also reduced by UCN3 overexpression, with and without PA treatment ($p<0.05$).

3T3-L1 adipocytes were transfected with UCN3 plasmid followed by PA treatment to elucidate the effect of UCN3 on the inflammatory pathway. UCN3 overexpression led to a significant decrease in TNF-α mRNA with and without palmitic acid treatment as

shown in Figure S6 in publication IV. UCN3 overexpression reduced IL6 mRNA in cells treated with PA. Interestingly, UCN3 overexpression, without concomitant treatment with PA, increases IL6 mRNA (Figure S6 in publication IV).

5.12 UCN3 overexpression increases glucose uptake

To further study the metabolic effects of UCN3, we studied the impact of UCN3 overexpression on glucose uptake and insulin signaling pathway in 3T3-L1 adipocytes with and without PA treatment (publication IV).

Insulin-stimulated glucose uptake was significantly increased by UCN3 overexpression in pre-adipocytes compared to PCMV transfected control cells ($p<0.01$) (Figure 4A in publication IV). A further increase in glucose uptake was observed in 3T3-L1 cells differentiated up to day 4 following UCN3 transfection. A similar increase in glucose uptake was also observed in UCN3 transfected mature adipocytes (differentiated for 8 days) ($p<0.05$) with and without PA treatment (Figure 4B in publication IV). The increase in glucose uptake with UCN3 overexpression was also observed at basal conditions without insulin stimulation (Figure S2 in publication IV).

Considering the enhanced glucose uptake following UCN3 overexpression, we next investigated the effect of UCN3 overexpression on the insulin signaling pathway. A significant increase in the phosphorylation AKT (at Ser^{473}) and ERK was observed, with a significant reduction in the phosphorylation of JNK was observed in response to UCN3 transfection at basal conditions (Figure S3 in publication IV).

The effect of UCN3 overexpression on the insulin signaling pathway markers was further studied in cells treated with PA without insulin stimulation. In cells exposed to PA, UCN3 overexpression led to an increase in the phosphorylation of ERK, whereas the phosphorylation of AKT or JNK was not affected (Figure 5 in publication IV). PA unexposed cells when cultured, the UCN3 overexpression led to an increase in the phosphorylation of AKT and ERK with a decrease in the phosphorylation of JNK.

6 Discussion

This thesis comprises four studies that demonstrate the role of UCN3 in obesity and T2D. These studies report for the first time 1) impairment of UCN3 levels in plasma and adipose tissue and normalization of SAT UCN3 expression levels following exercise in adults with obesity and T2D, 2) dysregulation of UCN levels in plasma and PBMCs from children with obesity, 3) metabolic stress with the exposure to palmitic acid and high glucose exacerbated ER stress and inflammation with concomitant dysregulation of UCN3 levels and, 4) enhanced insulin signaling and glucose uptake along with mitigated ER stress and heat shock response with UCN3 overexpression in 3T3L1 adipocytes. These results collectively suggest that circulating UCN3 is potentially involved in modulating meta-inflammation and stress responses in obesity. Impairments in UCN3 levels also indicate an adaptive response to metabolic insults and suggest the existence of compensatory mechanisms that might mitigate obesity-induced stress responses.

6.1 Study I: UCN3 expression is impaired with obesity and T2D in overweight adult people (publication I)

The principal findings are 1) circulating UCN3 levels are reduced in overweight compared to normal-weight participants and increased in T2D compared to overweight non-diabetic subjects, 2) there are independent associations between UCN3 plasma levels and glycemic markers (FPG and HbA_{1c}), 3) there is a significant reduction in UCN3 expression in SAT following a monitored three-month exercise regimen.

With insulin resistance, feedback inhibition of insulin secretion is impaired along with dysregulation of central and peripheral endocrine and inflammatory pathways (Muscelli et al. 2001; Guillausseau et al. 2008). Van Der Meulen has reported the co-release of UCN3 with insulin from β-cells which has a paracrine action on somatostatin releasing delta cells to limit insulin release (van der Meulen et al. 2015). Hence, dysregulated UCN3 expression in obesity and T2D may influence insulin secretion in these conditions. We have observed independent associations between

UCN3 plasma levels and glycemic markers, further suggesting a role of UCN3 in the regulation of glucose homeostasis. Additionally, a significantly higher circulating UCN3 concentration in overweight people with T2D compared to non-diabetic overweight individuals highlights a possible role of UCN3 in the pathogenesis of T2D.

The variations in the expression of UCNs depend on tissue and disease. In line with this, UCN1 levels in plasma and heart have been reported to be increased in the early stages of heart failure and decreased with HF severity, reflecting attenuated cardioprotection (Ng et al. 2004). Further, UCN1 expression in the heart decreased in people undergoing bypass surgery, with and without diabetes (Chen-Scarabelli et al. 2014a). Also, plasma CRF levels increased during gestation from 8 weeks to 36 weeks exponentially (Pepels et al. 2010b). On the contrary, UCN2 and UCN3 were not secreted by the placenta.

UCN3 gene transfer and targeting CRFR2 with agonists UCN3 or UCN2 enhances cardiac function glucose control and reduces the liver fat content. Further, UCN3 has been established to be present in various tissues such as the intestine, stomach, kidney, brain, pancreas, and muscle, although the source, concentrations, and UCN3 forms need to be investigated (Brar, Jonassen, Egorina, Chen, Negro, Perrin, Mjos, et al. 2004; Huising and Vale 2009). The differential pattern of UCN3 observed in SAT and plasma suggests an insignificant contribution of secretion from SAT to the circulating UCN3 levels. Due to the opposite pattern of UCN3 in SAT and plasma validated through mRNA and protein levels, we can disregard the uptake of UCN3 by adipose tissues. However, the impaired expression of UCN3 levels in SAT and plasma supports its role in the feedback loop connecting insulin and glucose. This feedback loop seems impaired in overweight individuals with and without diabetes, who also have impaired glucose homeostasis.

Significant correlations between UCN3 and GLP-1 were observed, further supporting the link between UCN3 and regulation of insulin secretion. Also, UCN3 secretion has been demonstrated in previous research, stimulated by high glucose and GLP-1 in β-cell cultures (Li et al. 2007). Furthermore, we have observed significantly increased UCN3 levels in SAT from non-diabetic overweight subjects compared to normal-weight participants. On the other hand, UCN3 levels were decreased in overweight with T2D compared to the non-diabetic overweight group. This decrease agrees with earlier studies showing reduced UCN3 levels in β-cells from diabetic

subjects (van der Meulen et al. 2015). Interestingly, we also observed a strong correlation of UCN3 with TGL and visfatin in plasma levels implying systemic metabolic dysregulation due to increased BMI and glucose levels in our study cohort. Visfatin is an adipokine with insulin-mimetic and pro-inflammatory effects (Fukuhara et al. 2005). Hyperglycemia has been reported to increase visfatin levels (Haider et al. 2006). The positive association between UCN3 and visfatin contributes to the complex relationship between insulin secretion, insulin resistance, adipokines, and hypertriglyceridemia.

The increased SAT UCN3 expression observed in overweight participants may indicate an adaptive mechanism to mitigate metabolic stress and meta-inflammation caused by obesity. Diabetes, in combination with obesity, further increases glucolipotoxicity, disturbs homeostasis, and leads to saturation of adaptive systems, which may be the cause of decreased UCN3 levels in SAT in overweight diabetics compared to non-diabetic overweight people. We have previously reported similar patterns in human SAT for heat shock response systems (Khadir, Kavalakatt, et al. 2018a). Moreover, in our cellular experiments with murine adipocytes, we demonstrated a marked reduction of UCN3 with concomitant exposure to macrophage-conditioned media, raising a possibility that inflammation of adipose tissue in diabetes may decrease UCN3 expression.

Physical exercise has been demonstrated to reduce the risk of metabolic diseases by regulating stress and inflammatory responses (Kawanishi et al. 2010). Our study showed that a monitored three-month exercise regimen significantly increased plasma UCN3 levels in the non-diabetic overweight group with a concomitant enhancement in $VO_{2\ max}$ and reduction in insulin levels, PBF, and waist circumference. We and others have reported the positive impacts of regular exercise on non-diabetic individuals compared with people with T2D (Khadir, Kavalakatt, Madhu, et al. 2018; Blaak et al. 2000; Khadir, Kavalakatt, et al. 2018a). However, the impact of exercise on circulating UCN3 levels was not parallel to an effect on SAT UCN3, as SAT UCN3 expression was decreased in response to exercise training. The differential regulation of plasma and SAT UCN3 in response to exercise training raises questions about the role of UCN3 in different tissues. Although a limited number of studies report the effects of exercise on UCN3 in health and disease, previous studies have suggested a possible link between exercise and the responsiveness of the hypothalamic-pituitary-

adrenal axis, CRF system activation, and cortisol secretion (St-Pierre and Richard 2013; Yanagita et al. 2007).

6.2 Study II: Levels of UCNs are impaired in the plasma from overweight children (publication II)

children (publication II)

The principal findings of this study are: 1) In overweight children, there is a significant impairment in UCN levels which is less significant in obese children. 2) UCN2 and UCN3 have a different expression pattern than UCN1 levels suggesting a possible compensatory effect. 3) UCN1 and UCN2 are independently associated with UCN3 levels.

In line with previous studies, we have observed expected impairments in glycemic and lipid profiles along with elevated levels of leptin, other adipokines, and inflammatory markers including TNF-α, NGAL, RBP4, ZAG, and sICAM1 in overweight and obese children in comparison to normal-weight children (Singer and Lumeng 2017; Patel et al. 2011). There is also a significant decline in adiponectin levels. These data highlight disturbances in food intake, energy balance, glucose, lipid metabolism, and impaired metabolic stress response in overweight children. Furthermore, we have observed elevated ALT levels and a trend for increased γ-glutamyl transferase (γGT) with increased body weight. Augmented ALT and γGT have been reported as indicators for the development of pre-diabetes and T2D in healthy individuals. Both markers are also considered surrogate markers for non-alcoholic fatty liver disease, liver dysfunction, and metabolic syndrome (Patel et al. 2011). Our data show a significant increase in ZAG levels with increased body weight. ZAG has previously been reported involved in the stimulation of lipolysis and reduction of body fat in mice, while ZAG levels are increased with the onset of type 2 diabetes (Vanni et al. 2009).

The CRF family plays an important role in autocrine and paracrine regulation (van der Meulen et al. 2015; Kavalakatt et al. 2019b) of various physiological processes such as food intake, energy balance (Fekete and Zorrilla 2007; Roustit et al. 2014a;

Chao et al. 2012; Kuperman et al. 2010; Chen et al. 2010), stress response (Fekete and Zorrilla 2007), and inflammation (Temur et al. 2016; Jamieson et al. 2011a). Our data demonstrate dysregulation of plasma levels of UCNs with increasing weight in children. Interestingly, significant impairments were observed in overweight children but not in obese children.

These data indicate an important role of UCNs during early periods of increased BMI and development of metabolic syndrome before the development of frank obesity; this also raises the possibility that changes in UCNs function as an early response mechanism in trying to combat increasing body weight and insulin resistance. In line with this hypothesis, Jameison et al. have demonstrated that UCN3 transgenic mice have a metabolically favorable phenotype and resist obesity and hyperglycemia following a high-fat diet (Jamieson et al. 2011a). CRFR2 agonists UCN2 and UCN3 have been reported to have beneficial effects on cardiovascular function, glucose control, reduction in liver fat, and weight loss (Fekete and Zorrilla 2007). Furthermore, Alarslan et al. have reported that circulating UCN3 levels are increased in overweight people with recent onset of T2D compared with healthy controls (Alarslan et al. 2020). In line with this, in the previous study (Study I), we demonstrated that UCN3 is differentially expressed in plasma and SAT from overweight adults.

These results suggest that circulating UCN3 regulates feeding and energy homeostasis and is potentially involved in modulating meta-inflammation in obesity. The positive correlation supports the latter notion observed in our study between UCN3 and the TNFα, RBP4, and ZAG proteins. Hence, the increased levels of UCN2, particularly UCN3, observed in our study may reflect a compensatory mechanism to suppress further food intake in overweight subjects. CRFR2 knock-out mice have been found to eat larger meals instead of smaller meals consumed in CRFR2 agonist-treated rats (Tabarin et al. 2007). Also, UCN2 peptide decreases nocturnal feeding and causes rats to consume smaller meals less frequently (Tabarin et al. 2007). Accordingly, increases in UCN2 and UCN3 in overweight may be beneficial in regulating meal satiation.

In previous studies, UCN1 has been associated with inflammatory and hypertensive conditions. For instance, circulating maternal and fetal UCN1 levels are increased in gestational hypertension, pre-eclampsia, and other hypertensive disorders

during pregnancy (Florio et al. 2006). In contrast, decreased plasma UCN1 levels are observed in intrahepatic cholestasis in pregnancy (Zhang et al. 2015). As UCN1 has vasoactive properties, elevated levels may represent an adaptive stress response (Florio et al. 2006). UCN1 also increases neuronal activation related to reducing ghrelin secretion and food intake (Yakabi et al. 2011). Furthermore, increased plasma UCN2 following UCN2 gene transfer improves total body glucose disposal in insulin-resistant HFD fed mice (Kim et al. 2019a). However, people with non-ischemic cardiomyopathy exhibit an 8 fold increase in plasma UCN2 level compared with healthy controls (Tsuda and Takefuji 2017). Also, serum UCN2 levels in hypertensive patients are significantly higher when compared with non-hypertensive patients (Aslan and Aytekin 2020). These studies collectively demonstrate a context-related status for UCNs.

In contrast to the UCNs, reduced circulating levels of CRF have been reported in people with T2D (Hashimoto et al. 1993). Increased plasma CRF may partly be responsible for stress-induced ACTH secretion (Hashimoto et al. 1989). Consistent with this, a reduction in plasma CRF levels has been observed with decreased neurotoxicity in an antioxidant-treated Alzheimer's disease rat model (Ooi et al. 2020). Hence, reduced CRF levels are mostly associated with dysregulated homeostasis and disorders. In our study, circulating CRF levels are not affected by increased BMI in children; this may result from the relatively lower cellular and metabolic stress in this age group than adult individuals who frequently exhibit insulin resistance and other metabolic disorders.

Our data reveal striking sex differences in the levels of UCNs between boys and girls. Although UCN1 is lower in girls, UCN2 and UCN3 are higher than boys. Comparable observations have been previously reported in CRFR2- and CRFR1-deficient mice which exhibit sexually dichotomous anxiety-like behavior (Bale et al. 2002). In CRFR2 knock-out mice, impaired glucose tolerance develops in male mice but not in female mice fed with a chow diet. Hence, CRFR dysregulation is a sexually dimorphic factor associated with the development of diabetes and other metabolic syndromes (Paruthiyil et al. 2018). As the HPA axis interacts with many other physiological pathways, the changes in endocrine function are also sex-specific and age-dependent. The implication of such observations on the role of UCNs and their

receptors in sex-related vulnerability or resistance to metabolic diseases requires further investigation.

Spexin is another neuropeptide that has similar functionalities as the CRF neuropeptide family. We have recently reported that circulating levels of Spexin are decreased with obesity and diabetes in adults and inversely correlate with adiposity indicators (BMI, waist, and hip circumference), blood pressure, and lipid markers (LDL, TG, and TC), but positively correlated with HDL levels (Khadir et al. 2020). These data together support a role for Spexin in energy metabolism and weight regulation with a potential link to obesity and diabetes (Lv et al. 2019). However, we find no significant changes in overweight and children with obesity compared with matched normal-weight controls. In line with this finding, Hodges et al. (Hodges et al. 2018) did not report any variation in Spexin levels in adolescents with obesity or diabetes, and there was no correlation with body composition or blood measurements, indicating that Spexin may not act as a metabolic regulator in adolescents.

6.3 Study III: Levels of UCNs are impaired in the PBMCs from overweight children (publication III)

The main findings of this study were: 1) A significant reduction in transcript levels of UCN1, UCN3, and CRF with increased BMI, 2) A significant association of UCN3 with UCN1, UCN2, and CRF, 3) A decreased expression of UCN3 in THP1 cells treated with high glucose, palmitate, or their combination and a decreased expression of CRF in THP1 cells treated with high glucose or palmitate for a short duration of 4 hours, 4) and an increase in UCN2, UCN3 and CRF mRNA expression with a concomitant increase in mRNA expression of inflammatory and ER stress markers in THP1 cells exposed to palmitate or a combination of high glucose and palmitate for 24 h, highlighting possible crosstalk between cellular stress pathways and CRF family of neuropeptides.

The CRF family of neuropeptides are expressed in immune cells, including lymphocytes, macrophages, and immunological tissues, including the spleen and thymus (Fekete and Zorrilla 2007). CRFR2 agonists can modulate corticosterone levels, the main pituitary adrenocortical hormone controlling stress, adaptation, and

metabolism, in a dose-dependent manner. This modulation depends on the synthesis and release of CRF, as observed with a reduction of plasma corticosterone levels with decreased CRF and increased or no change with increased CRF levels (Fekete and Zorrilla 2007; Suda et al. 2004). Further, UCN1-3 expression is also induced by elevated glucocorticoid levels (Tillinger et al. 2013; Huising et al. 2011; Kageyama et al. 1999). These observations highlight the critical role of CRF peptides in modulating stress-induced hormonal and behavioral responses. Such elevated glucocorticoid levels aggravate metabolic stress due to obesity, reduce insulin secretion, increase insulin resistance, and lower peripheral glucose uptake (Livingstone et al. 2000; Rizza, Mandarino, and Gerich 1982). In this study, we have observed reduced CRF, UCN1, and UCN3 in PBMCs with increased body weight, highlighting a potential feedback mechanism to curb the progression of obesity in children by reducing glucocorticoids.

Elevated UCN2 expression in the hypothalamus has been associated with a preference for high-fat food in rats (Alsiö et al. 2009). However, lower UCN2 expression in blood cells from children has been correlated with increased consumption of food with high-fat content when compared with low or intermediate fat intake (Priego et al. 2015). Interestingly, no changes in UCN2 transcript levels were observed in our cohort with increased body weight.

The pro-inflammatory role of UCN1 and CRF under environmental stress conditions has been highlighted in several studies. Elevated UCN1 and CRF expression levels were reported in intestinal macrophages from ulcerative colitis patients (Saruta et al. 2004), with increased UCN1 expression correlating with the severity of inflammation (Uzuki et al. 2001). Further, UCN1 also stimulates the production of inflammatory markers IL1β and IL6 in PBMCs (Kohno et al. 2001). However, in our study, there was a significant decrease in UCN1 expression with increased levels of inflammatory marker TNFα and body weight in overweight children. This opposite pattern between UCN1 and inflammatory marker could indicate a protective mechanism to lower the elevated inflammatory stress with obesity.

The anti-inflammatory role of UCN2 has been revealed through the decreased expression of inflammatory markers in macrophages induced with LPS (Chatzaki et al. 2003). Consistent with this study, Tsatsanis has demonstrated that UCN2 lowers macrophage apoptosis and IL6 production (Tsatsanis et al. 2005a). Although the

differences in UCN2 transcript levels with BMI were statistically nonsignificant in our cohort as well as with a short-term PA treatment, there was a marked increase in UCN2 levels along with elevated inflammatory and ER stress markers in THP1 cells treated with palmitate with or without high glucose for a longer duration. In keeping with findings in earlier studies, increased UCN2 levels in response to 24 h treatments with palmitate or the combination of high glucose and palmitate may indicate a protective mechanism to mitigate inflammatory response and maintain homeostasis (Simpson et al. 2020b).

Metabolic stress in children at early stages of obesity is better controlled by related stress responses than in adults, where chronic stress can lead to aggravated metabolic responses. In line with this, short-term treatment of THP1 cells with high glucose or palmitate lowered CRF and UCN3. Spexin and UCN3 expression was reduced in response to short-term exposure to a combination of high glucose and palmitate. On the other hand, treatment for a longer duration, especially with PA, significantly increased CRF, UCN2, and UCN3 mRNA levels in THP1 cells; such increases were also observed with elevated mRNAs for inflammatory and ER stress markers. These stress markers did not increase with chronic high glucose treatments suggesting that stress responses were not stimulated by glucose, as demonstrated by decreased expression of Spexin and CRF and no change in mRNA levels of other examined neuropeptides in response to 24 h exposure to high glucose.

Interestingly, we have observed a significant increase in UCN3 plasma levels in overweight children and decreased plasma UCN3 in non-diabetic overweight adults (Kavalakatt et al. 2019a), keeping with the trend seen in UCN3 levels in PBMCs. However, in overweight adults with T2D, circulating plasma UCN3 levels were increased (Kavalakatt et al. 2019a). These varying patterns of CRF neuropeptides with age and severity of metabolic stress highlight their transition between protective and pro-inflammatory roles with obesity in children compared with adults.

Spexin is another neuropeptide that has been implicated in the regulation of weight and energy metabolism (Lv et al. 2019). Plasma Spexin level is high in adults with obesity and diabetes and can be normalized with physical activity (Khadir et al. 2020). However, Hodges et al. have shown the lack of an association between Spexin and body composition and no significant differences in its levels with obesity or diabetes in adolescents (Hodges et al. 2018).In line with this finding, there were no marked

changes in Spexin levels with increased BMI in our cohort. Hence, Spexin may not be a key metabolic regulator of obesity in children but might have an age-dependent role.

Our study demonstrates an impaired expression of CRF neuropeptides in PBMCs obtained from overweight and obese children with possible crosstalk between cellular stress response pathways. This dysregulation in PBMC neuropeptide levels indicates an early adaptive response to metabolic insults and suggests that compensatory mechanisms mitigating obesity-induced stress response may exist.

6.4 Study IV: UCN3 overexpression reduces ER stress and heat shock response in adipocytes (publication IV)

We show that overexpression of UCN3 in 3T3-L1 adipocytes attenuates heat shock and ER stress responses and mitigates apoptosis and inflammation. Overexpression is also associated with enhanced glucose uptake in murine pre-adipocytes and mature adipocyte with concomitant improvement in insulin signaling through upregulated AKT and ERK phosphorylation and downregulated JNK phosphorylation. Comparable benefits are observed with the treatment of palmitate as a fatty acid stressor. Re-analyses of Study I data demonstrate negative associations between levels of UCN3, HSP60, and GRP78 in non-diabetic human plasma, as well as in 3T3-L1 adipocytes differentiated in the presence of MaCM. Thus, UCN3 may play a favorable role in metabolic disorders, including obesity and diabetes.

UCN3 overexpression in skeletal muscle has been reported to enhance glucose uptake and insulin signaling through upregulation of GLUT1, GLUT4 protein expression, and activation of the AMPK signaling pathway (Roustit et al. 2014a). Further, UCN3 gene transfer has improved glucose control, reduced liver fat, and aided in weight loss (Giamouridis et al. 2018a). In line with this, with UCN3 overexpression, we have observed improved glucose uptake in pre-adipocytes and adipocytes. A previous study has demonstrated the favorable phenotype of $UCN3^{+}$ transgenic mice with protection from metabolic challenges, including obesity and hyperglycemia; this could be due to the augmented glucose and fatty acid metabolism (Jamieson et al. 2011a). Interestingly, UCN3 KO mice also demonstrate improved insulin sensitivity and favorable metabolic resilience with a high-fat diet.

Further, aged KO mice showed enhanced glucose homeostasis compared to WT mice (Li et al. 2007; Chao et al. 2012). Hence, any disturbance in UCN3 levels, overexpression, and depletion, leads to metabolic protection. Stimulation of muscle CRFR2 increases muscle mass and improves glucose homeostasis (Jamieson et al. 2011a; Hinkle et al. 2003); this improvement in the metabolic state by muscle CRFR2 agonism could be due to UCN3 overexpression and its impact on the peripheral tissues. However, experimental data demonstrating this phenomenon are yet lacking.

Cellular homeostasis is maintained through intricate crosstalk between HSPs, ER stress, and inflammatory regulators (Kondo et al. 2011; Gregor and Hotamisligil 2011). HSP levels, particularly HSP72, are reduced with diabetes in humans (Kurucz et al. 2002). However, we have recently reported increased HSP expression in non-diabetic obese people both plasma and adipose tissue (Tiss et al. 2014; Khadir et al. 2016; Khadir, Kavalakatt, et al. 2018a); this suggests that HSP could maintain homeostasis in moderate metabolic stress related to obesity but not with the severe stress observed in diabetes.

When treated with high concentrations of palmitic acid, there is an expected increase in Heat shock and ER stress response along with apoptosis marker CHOP. Interestingly, UCN3 overexpression in adipocytes before palmitate exposure leads to reduced HSP levels, probably due to a lack of requirement for activation of a defense mechanism. Compared to the unexposed, the limited effect is observed on ER stress markers in response to UCN3 overexpression and palmitate treatment. A similar trend is observed in AKT, JNK, and ERK insulin signaling pathway markers. These may be due to excessive exposure to stress that overwhelms the cellular response systems. We and others have previously reported that expression of GRP78, the ER stress master regulator, is impaired with obesity and diabetes (Khadir, Kavalakatt, et al. 2018a; Tiss et al. 2014; Khadir et al. 2016). The significant reduction in apoptotic marker CHOP with UCN3 overexpression in the presence or absence of palmitate exposure suggests that UCN3 alleviates metabolic state through improved cellular stress response mechanisms.

Various metabolic disorders with inflammation, mitochondrial dysfunction, and apoptosis have been reported earlier (Li et al. 2012). Urocortins have been shown to exert anti-inflammatory effects via GSK and PI3K/AKT pathways (Wang et al. 2007).

Furthermore, UCNs are suggested to be cardioprotective through the MAPK pathway (Brar et al. 2002; Chen-Scarabelli et al. 2013; Barry et al. 2010). Accordingly, we have observed that the overexpression of UCN3 has anti-inflammatory effects with palmitate treatment as reflected by reduced TNFα and IL6 levels. However, with UCN3 overexpression, there is an unexpected increase in IL6 expression in the absence of palmitate treatment. IL6 is a cytokine with established pro-inflammatory functions. However, it also plays a role in non-inflammatory actions such as lipolysis, increased energy expenditure, and enhanced glucose disposal (Han et al. 2020). Short-term IL6 signaling has been reported to be cardioprotective in response to tissue damage and myocardial infarction. However, upon prolonged IL6 activation, this becomes pathogenic (Fontes, Rose, and Cihakova 2015). Accordingly, the decreased activation of cellular stress response mechanisms with UCN3 overexpression could be balanced by the upregulation of IL6.

We reanalyzed our data from non-diabetic adults (Publication I) and observed a significant negative association between plasma levels of UCN3, HSP60, and GRP78. Interestingly, this association is not observed in diabetic subjects. From Study I, we have also observed a correlation between UCN3 and RANTES in people with diabetes. In line with these observations, we report in this study that the co-culture of murine adipocytes with MaCM stimulates ER stress, HSPs, and CHOP levels and concomitantly reduces UCN3 expression. Co-culture with macrophage conditioned medium affects the cellular microenvironment and characterizes low-grade inflammation, as represented in obesity. Hence, the cellular microenvironment could play a role in UCN3 expression highlighting its role in adaptive responses and maintenance of homeostasis. Nevertheless, this effect is diminished under conditions of chronic stress.

7 Strengths and limitations

The main strength of this thesis work includes the enrollment of adults and children with varying BMI, which enabled us to study the impact of obesity as metabolic stress. The adult participants underwent a monitored exercise regimen (without dietary restriction) for three months as a lifestyle intervention to improve their health. We were the first to evaluate circulating levels of the CRF neuropeptides in plasma, PBMC, and subcutaneous adipose tissue from humans. We also reported the protective role of UCN3 overexpression in adipocytes for the first time, and this cellular level work is also endorsed by the adult human plasma data.

Some limitations, however, need to be considered. We could not access adult participants' visceral adipose tissue (VAT). VAT would have probably been more significant to the pathophysiology of obesity and T2D. As the participants' diet was not controlled, dietary factors might have also affected the UCN3 levels. The overweight, the diabetic group, was somewhat older than the healthy overweight adult group. The cross-sectional study design does not allow us to determine whether the attenuated neuropeptide levels contribute to the development of obesity. The low number of children enrolled in the normal weight, and overweight groups limit the power to evaluate correlations between neuropeptide levels and other clinical parameters. In addition, due to multiple comparisons, the statistical significances must be interpreted with caution, although we used the Bonferroni correction. No data regarding the children's family history, diet, or physical activity were collected. In addition, the measured levels of the neuropeptides in the plasma and PBMCs may not reflect their central and peripheral bioavailability. Knockdown experiments were not performed to validate further and assess the effect on the CRHR2 receptor in the studied cell models. Further insulin signaling pathway characterization and profiling could elucidate the role of UCN3 on adipocytes under conditions of obesity and diabetes.

8 Conclusions

CRF system has attracted immense research interest over the past decades as a critical player in the stress response system. Cumulative evidence highlights the role of altered function of the CRF system in the pathogenesis of mood, obesity, diabetes, and other related disorders. UCN3 is the most recently discovered neuropeptide of this system. Here, we provide evidence of the dysregulation of UCN3 in obesity and T2D, and we support its potential role as an early and modulable marker of metabolic-induced cellular stress.

Study I of this thesis revealed the association of UCN3 with markers of glucose metabolism and the differential alterations of UCN3 levels with obesity and T2D in human plasma and SAT. Moderate exercise training normalized SAT UCN3 expression.

Studies III and IV showed alterations in plasma and PBMC profile of the UCN neuropeptides with increased body weight in children. The marked changes observed in the plasma from overweight children were attenuated in obese children suggesting a possible compensatory mechanism for these neuropeptides to mitigate the progression of obesity and associated complications. UCN3 expression might be used as a biomarker for early detection of risk for weight gain in children.

In study II, overexpression of UCN3 attenuated markers for inflammation, apoptosis, ER stress, and HSR; these events were accompanied by enhanced glucose uptake and insulin signaling. The inflammatory microenvironment affected the expression of UCN. Increased UCN3 levels may exert possible protective effects against metabolic insults. However, the physiological effects of UCN3 are complex, requiring crosstalk between adaptive mechanisms both centrally and peripherally. Hence, a better understanding of the molecular mechanisms of UCN3 action would provide insight into its role in the pathophysiology of metabolic disorders.

Several pre-clinical studies have shed light on the therapeutic potential of the CRF family. Determination and validation of reliable biomarkers that could discover, monitor, and unravel stress-related disorders' pathophysiology are critical. Several studies have highlighted the role of the CRF family, including UCNs, as an integrator between physiological responses to stress and interaction between immune and neuroendocrine systems. The involvement of the CRF family in the pathogenesis, treatment, or management of stress-related disorders such as hypertension, congestive heart failure, obesity, diabetes, sleep apnea, inflammatory diseases, and other metabolic disorders raises major opportunities to target this system to develop novel pharmacological interventions toward the treatment of these common disorders.

9 Future perspectives

CRF system has attracted immense research interest over the past decades as a critical player in the stress response system. Over 100 patent claims were registered for small molecule CRF receptor antagonists and ligands, reinstating the significance of the CRF neuropeptide family. Cumulative evidence highlights the role of impaired CRF system function in the pathogenesis of mood, obesity, diabetes, and other related disorders. This understanding could aid in better strategies to discover and monitor progression and therapies for stress-related disorders.

The technological advancements could provide novel possibilities to the CRF system. Improvements in cryo-electron microscopy would resolve receptor interactions and signal transduction mechanisms(Kühlbrandt 2014); this would enable the design of agonists and antagonists to overcome the limited efficacy of various small molecules that did not succeed in the clinical trials. New possibilities of the CRF system were unraveled with animal genetic models. Moreover, a broader spectrum of genetic tools is necessary to investigate the impact of the CRF system on stress response and how stress affects them. The complexity of CRF and Urocortins signaling has been highlighted through several in-vitro studies. In-vivo studies and organoid cultures could better understand peptide release and receptor activation; this raises the question of the link between molecular and cellular levels translating to behavioral levels, which could promote adaptations. The current technological advancements and international collaborations would promote further understanding of brain, behavior, and stress-related disorders (Grillner et al. 2016).

Despite the complex system, several pre-clinical studies have shed light on the therapeutic potential of the CRF family. Further efforts, however, were stalled due to inconclusive clinical trials. However, with the current advancements, these studies need to be reconsidered. Inclusion criteria for clinical trials should also consider assessments of genetic profiles and their possible associations with the stress response pathways. Several directions of the CRF family could be added with promising future perspectives, such as interactions with other systems.

Nevertheless, this demonstrates the complexity of the stress-related systems and hence, the requirement of more detailed studies, including appropriate animal models to identify the various stress-affected systems. Determination of reliable biomarkers

which could discover, monitor, and unravel the pathophysiology of stress-related disorders is critical. The CRF family of neuropeptides could serve as biomarkers, in combination with genetic profiling, could lead to personalized therapy opening new avenues of diagnosis and treatment strategies of stress-related metabolic diseases (Holsboer and Ising 2009).

Several studies have shed light on the role of the CRF family, including Urocortins as an integrator between physiological responses to stress and interaction between immune and neuroendocrine systems. Its involvement in metabolic diseases including obesity, diabetes, sleep apnea, inflammatory diseases, and other disorders is promising pharmacological intervention areas.

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