ROLE OF GHRELIN IN METABOLIC AND MUSCLE DISORDERS

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CERTIFICATE OF ORIGINALITY

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_____ (Signed)

UGWU Felix Nnaemeka (Name of student)

Dedication

This thesis is dedicated to my lovely wife and adorable children

Abstract

Ghrelin gene products namely unacylated ghrelin (UnAG), acylated ghrelin (AG) and obestatin are key metabolic molecules being studied for their therapeutic potentials. The aim of this thesis was to investigate the role of ghrelin axis in cardiometabolic disorders including metabolic syndrome (MetS), hypertension, central obesity and pressure ulcer. The first cardiometabolic disorder being studied in this thesis was MetS which is a cluster of cardiometabolic risk factors including central obesity, elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, and reduced high-density lipoprotein cholesterol (HDL-C), that predisposes individuals to cardiovascular diseases and type 2 diabetes mellitus. The influence of the interaction of central obesity with the other MetS components including blood pressure, fasting blood glucose, triglycerides, and HDL-C on circulatory UnAG, AG, obestatin, growth hormone (GH), insulin-like growth factor-1 (IGF-1) and nesfatin-1 was examined in 133 adults. The results of this study revealed that 1) obestatin, obestatin/ghrelin ratios, and GH were influenced by the interaction of central obesity with the other MetS risk factors 2) central obese subjects with or without the cluster of the other MetS risk factors had higher obestatin level but lower GH level 3) UnAG, AG and GH were lower in subjects with the cluster of MetS risk factors whereas obestatin was higher in subjects with the cluster of MetS risk factors and 4) ghrelin gene products and GH were correlated with the risk factors of MetS. Besides providing evidence for prioritizing central obesity as the main MetS risk factor, this study clarified the synergistic or differential role of central obesity among the other MetS risk factors. In the second study, obesity and hypertension, two independent cardiometabolic risk factors that increase morbidity and mortality, were investigated. In

this study, the interacting influence of central obesity with hypertension on UnAG, AG, obestatin, ghrelin/obestatin ratio, and GH. Fasting plasma abundances of UnAG, AG, obestatin and GH were determined in 387 female adults. Blood samples were selected based on a 2 x 2 factorial design for central obesity and hypertension from an archived sample pool of 1492 participants who were previously screened for MetS. Our results revealed a significant interaction effect of hypertension with central obesity on obestatin. Obestatin was found to be significantly higher in central obese-only subjects when compared to subjects with neither hypertension nor central obesity and central obese subjects with hypertension. UnAG, AG, total ghrelin, ratios of UnAG/obestatin, AG/obestatin, total ghrelin/obestatin and GH were higher in subjects with neither hypertension nor central obesity when compared to central obese subjects with hypertension. This thesis is the first to examine the complex interaction of hypertension with central obesity on ghrelin axis. Our results suggest that ghrelin axis might play unique role in the regulation of blood pressure and control of body fat mass. Cardiometabolic risk factors of MetS negatively affect overall quality of life, Exercise and pharmacological interventions have been recommended to arrest the progression of the cardiometabolic risk factors of MetS. In the third study, we examined the effects of 1-year yoga training on circulatory peptides associated with ghrelin axis including UnAG, AG, obestatin, GH and insulin as well as homeostatic model assessment (HOMA) indices and disposition index in adults with MetS. Sera and data of 79 MetS subjects who had participated in a 1-year program of Hatha yoga exercise training or served as non-exercise control were analyzed. Our results revealed a significant interaction effect of group and time on UnAG, total ghrelin, obestatin and GH, but not AG and insulin. According to our

results, UnAG and total ghrelin tended to be higher, GH was higher whereas obestatin was lower in the yoga group when compared to control group after the 1-year experimental period. Furthermore, our results demonstrated that AG was lower in the yoga group when compared to control group whereas insulin, HOMA indices and disposition index were unaltered at the end of the 1-year experimental period. These findings suggest that 1-year yoga training alters circulatory ghrelin gene products and GH in adults with MetS. The study supports a role of ghrelin axis in the pathogenesis of MetS. Poor circulation, a consequence of hypertension, is aggravated by central obesity, dyslipidaemia, and diabetes. Poor circulation resulting from cardiometabolic disorders reduces the nutrients delivered to skeletal muscle cells leading to apoptosis, necrosis and oxidative stress which are characteristics of pressure. Pressure ulcers which are damages to the skin and underlying tissue are associated with increased morbidity and mortality particularly in the aged and individuals with restricted movements. UnAG, the predominant form of circulating ghrelin, has been shown to protect myotubes from cell death which is a known attribute of pressure ulcer. In the fourth study, we investigated whether UnAG could protect skeletal muscle from pressure-induced deep tissue injury by abolishing necroptosis and apoptosis signaling and whether these effects are mediated by SIRT1 pathway. Fifteen adult Sprague Dawley rats were randomly assigned to receive saline or UnAG with or without EX527 (a SIRT1 inhibitor). Animals underwent two 6-hour compression cycles with 100 mmHg static pressure applied over the mid-tibialis region of the right limb whereas the left uncompressed limb served as the intra-animal control. Muscle tissues underneath the compression region and at the similar region of the opposite uncompressed limb were collected for analysis. Our results showed that

UnAG 1) attenuated the compression-induced muscle pathohistological alterations including rounding contour of myofibers, extensive nucleus accumulation in the interstitial space, and increased interstitial space 2) abolished the increase in necroptosis proteins including RIP1 and RIP3 and 3) attenuated the elevation of apoptotic proteins including p53, Bax and AIF in the compressed muscle. The protective effects of UnAG were diminished in rats co-treated with EX527. Our results demonstrated that UnAG protected skeletal muscle from compression-induced injury and that the myoprotective effects of UnAG on pressure-induced tissue injury are associated with SIRT1 signaling. These findings have important implications in the prevention and treatment of pressure ulcer.

Conclusively, the current thesis has demonstrated that obestatin and the ratios of ghrelin gene products were influenced by the interaction of central obesity with the other four MetS risk factors as well as the interaction of hypertension with central obesity. UnAG and AG decreased in the presence of MetS risk factors whereas obestatin increased in the presence of MetS risk factors. 1-year yoga intervention, a non-pharmacological treatment, decreased obestatin but tended to increase UnAG. Finally, the action of UnAG in abolishing pressure ulcer-related apoptosis and necroptosis as demonstrated in thesis has proved that UnAG may serve a future therapeutic target in the management of pressure ulcers. Taken together, this thesis suggests that ghrelin axis is uniquely affected by biochemical pathways related to cardiovascular and energy-related disorders and might play indispensable roles in cardiometabolic disorders.

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List of abbreviations

AACE	American Association for Clinical Endocrinology
ADA	American Diabetes Association
AG	Acylated ghrelin
AHA	American Heart Association
AIF	Apoptosis Inducing factor
Akt	Protein kinase B
AMA	American Medical Association
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of Variance
AP	Action potential
ApoB	Apolipoprotein B
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B cell leukemia/lymphoma-2
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BP	Blood pressure
$CaCl_2$	Calcium chloride
CCK	Cholecystokinin
DBP	Diastolic blood pressure
DECODE	Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria ir
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
DTI	Deep tissue injury
ECF	Extracellular fluid
EGIR	European Group for the Study of Insulin Resistance

ELIZA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBG	Fasting blood glucose
G-6-P	Glucose-6-phosphate
GDP	Gross domestic product
GEE	Generalized estimating equations
GFR	Glomerular filtration rate
GH	Growth hormone
GHIH	Growth hormone-inhibiting hormone
GHRH	Growth hormone-releasing hormone
GHS	Growth hormone secretagogue
GHSR	Growth hormone secretagogue receptor
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GnRH	Gonadotropin-releasing hormone
GOAT	Ghrelin O-acyltransferase
HDL-C	High-density lipoprotein cholesterol
HIF2α	Hypoxia inducible factor 2 alpha
HOMA	Homeostatic model assessment
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HRP	Horseradish Peroxidase
HSD	Honestly significant difference
I/R	Ischemia - reperfusion
ICV	Intracerebroventricular
IDF	International Diabetes Federation
IFG	Impaired fasting glucose

IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor-1
IGF-BP	Insulin-like growth factor-binding protein
IGT	Impaired glucose tolerance
IR	Insulin resistance
JAK - STAT	Janus kinase - signal transducers and activators of transcription
LDL-C	Low-density lipoprotein cholesterol
MAFbx	Muscle atrophy F-box
MAPK	Ras-mitogen-activated protein kinase
MetS	Metabolic syndrome
mRNA	Messenger ribonucleic acid
mTORC2	Mammalian target of rapamycin complex 2
NCEP: ATPIII	National Cholesterol Education Program - Third Adult Treatment Panel
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
NPUAP	National Pressure Ulcer Advisory Panel
NREM	Non-rapid eye movement
NRFNO	No MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity)
NRFO	No MetS risk factors except central obesity
NUCB2	Nucleobinding 2
OD	Optical density
p53	Tumor protein 53
PARP-1	poly(ADP-ribose)polymerase-1
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene difluoride

RFNO	Cluster of the four MetS risk factors without central obesity
RFO	Cluster of the four MetS risk factors plus central obesity
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RIP3	Receptor-interacting serine/threonine-protein kinase 3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIGN	Scottish Intercollegiate Guidelines
SIRT1	Sirtuin 1
SPSS	Statistical Package for the Social Sciences
SST	Somatostatin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TNFα	Tumor necrosis factor alpha
UCP1/2	Uncoupling protein 1 / 2
UnAG	Unacylated ghrelin
US	United States of America
WC	Waist circumference
WHO	World Health Organization

Chapter 1. Introduction

It is generally accepted that muscle plays significant role in metabolic disorders by modifying the homeostasis of postprandial energy metabolism. The symptoms of metabolic disorders differ among people, with some individuals experiencing mild symptoms that are manageable with lifestyle changes, and others experiencing severe symptoms with life-threatening symptoms. Multiple symptoms of metabolic disorders including lethargy, progressive weight loss and jaundice[1] synergistically affect overall quality of life. Metabolic syndrome (MetS) which is a cluster of risk factors including central obesity, dyslipidemia, hyperglycemia, and elevated blood pressure[2] was identified as 'the 21st century epidemic' approximately a decade ago[3]. MetS is a global public health problem with approximately 1.4 billion people being affected by MetS in 2015 alone[4]. The increasing prevalence of MetS among all ethnic groups, age brackets and both genders has stimulated interest in the medical field.

Although MetS was first described by Raven in 1988, the main risk factor of MetS, central obesity, was identified almost two decades later by International Diabetes Federation (IDF). It has been estimated that half of the world's adult population may be affected by central obesity by 2030[5]. Rising prevalence and financial cost have transformed obesity from a mere syndrome to 'a disease with multiple organ-specific consequences'[6]. Since the identification of central obesity as the primary risk factor of MetS, several researchers have adopted IDF diagnostic tool in MetS studies. Moreover, strong associations between central obesity and several metabolic disorders including hypertension, insulin resistance and Type 2 diabetes Mellitus have been identified[7]. Although MetS has been discussed for

approximately 3 decades, the definition of MetS remains elusive as several factors are known to influence MetS. Given this uncertainty, studies investigating the interaction of central obesity and the cluster of the other MetS risk factors including dyslipidemia, hyperglycemia, and elevated blood pressure are important to clarify the role of central obesity in MetS.

Besides central obesity, raised blood pressure (hypertension) is considered an important MetS risk factor. A recent study identified elevated blood pressure in adults as a prevalent MetS risk factor, second only to central obesity[2]. The finding was corroborated by an extensive study of 19·1 million adults in 200 countries, reporting increasing prevalence of elevated blood pressure from 594 million in 1975 to 1·13 billion adults in 2015[8]. Independent studies have suggested a link between central obesity and elevated blood pressure, but studies addressing the interaction of central obesity and elevated blood pressure are relatively rare. Investigations of the interaction of central obesity and hypertension is needed to understand associated signaling pathways and unravel novel targets that could preserve or restore physiological functions in subjects with energy-related disorders.

Engagement in physical activities is known to maintain health since time immemorial. However, the type and duration of physical activity remain a matter of debate. Yoga exercise which mainly consists of postural changes and breathing exercises is believed to maintain cardiovascular and musculoskeletal health among adherents[9]. Existing recommendations have been proposed for the supplementary use of yoga for the management of heart diseases including coronary heart disease, heart failure and cardiac dysrhythmia[10]. The mechanisms

by which yoga modify physiological systems are not entirely known. More so, studies focusing on the long-term physiological effects of yoga are lacking in the literature. Prior research on short-term changes in MetS risk factors after yoga intervention have been demonstrated[11]. Whereas several short-term studies have reported beneficial effect of yoga exercise on MetS risk factors, a long-term study has recently demonstrated that among MetS risk factors, yoga intervention successfully decreased only central obesity, as measured by waist circumference[2]. There is a paucity of data in the literature regarding the effect of long-term yoga exercise on metabolic peptides, especially ghrelin gene products and growth hormone. Also, there is a dearth of evidence with reference to the effect of long-term yoga on β-cell function and insulin resistance, as the gold standard for interpretation of insulin secretion and resistance, the hyperinsulinemic-euglycemic clamp technique, is both laborious and capital intensive. To this end, mathematical methods including homeostatic model assessment (HOMA) indices might be adopted as practical and safe tool for estimating β-cell function and insulin resistance in long-term yoga exercises[12].

Pressure ulcer refers to damage to the skin, muscle or bone principally caused by continuous pressure on the body[13]. Besides pain and financial cost induced by pressure ulcers, partial or permanent damage to muscle or bone is a known negative effect of several pressure ulcers. The management of pressure ulcer is mainly focused on preventive measures to arrest further tissue damage, since there is currently no cure for pressure ulcer. In a rat-model of pressure ulcer, resveratrol, a potent activator of SIRT1 has been demonstrated to prevent pathohistological damages in skeletal muscle[14]. This finding suggested resveratrol agonists or SIRT1 stimulators might be beneficial in pressure ulcer.

The growing awareness about metabolic and muscle disorders has stimulated widespread interest in the underlying mechanisms associated with energy metabolism. The vast metabolic roles and complexity of growth hormone and ghrelin gene products - unacylated ghrelin, acylated ghrelin and obestatin - suggest that metabolic disorders might be associated with these peptides, their sources, receptors or signaling pathways. More importantly, the past decade has witnessed a renewed importance in signaling molecules or pathways closely related to ghrelin axis. For instance, the co-localization of nesfatin-1 and ghrelin in human gastric cells was recently reported, suggesting a dual role of the gastric cells on stimulation and suppression of appetite[15]. Also, UnAG, the predominant form of circulatory ghrelin has been demonstrated to prevent cell death in various tissues including the skeletal muscle via unidentified mechanisms[16]. Furthermore, the controversial effects of obestatin on feeding and weight control[17] suggest that the physiological roles of obestatin remain to be defined and abnormal obestatin level might contribute to several energy-related pathologies. Metabolic disorders including MetS and pressure ulcer remain the leading cause of morbidity and mortality in both developed and developing countries. Collectively, understanding the role of ghrelin in metabolic and muscle disorders is important for effective management of energy-related disorders, as ghrelin axis is hypothesized to play fundamental role in metabolism.

Chapter 2. Literature review and significance and objectives of study

2.1. Metabolic disorders

2.1.1. Overview of metabolism

Metabolism refers to the sum total of energy reactions that occur in the body. Metabolic pathways that synthesize molecules via energy utilization are called anabolic pathways, whereas metabolic pathways that release energy by breaking down complex molecules are called catabolic pathway[18]. Examples of metabolic pathways include citric acid cycle, electron transport chain, oxidative phosphorylation, glycolysis and pentose phosphate pathway[19]. Complex carbohydrates, proteins and fats are broken down by enzymes and chemicals in the digestive tract. The end products of digestion, sugars, amino acids, fatty acids and glycerol, are used by the body as fuels or stored mainly in the liver, muscles and adipose tissues[20]. Adenosine triphosphate (ATP) is a nucleotide which transports chemical energy within cells for metabolism[21].

2.1.2. Definition, types and symptoms of metabolic disorders

Metabolic disorders are described as disorders that disrupt normal metabolism. Although most metabolic disorders are known to have genetic origins, dysfunctional organs or systems have also been demonstrated to cause metabolic disorders[22]. For instance, type 2 diabetes, a metabolic disorder, is known to be caused by diseases of the liver or pancreas.

According to US National Library of Medicine, metabolic disorders are principally classified into acid-base imbalance, bone metabolic diseases, metabolic brain diseases, calcium metabolism disorders DNA repair-deficiency disorders, glucose metabolism

disorders, hyperlactatemia, iron metabolism disorders, lipid metabolism disorders, malabsorption syndromes, metabolic syndrome X, inborn errors of metabolism mitochondrial diseases, phosphorus metabolism disorders, porphyrias, proteostasis deficiencies, metabolic skin diseases, wasting syndrome and water-electrolyte imbalance. In general, the symptoms of metabolic disorder are classified into four types namely acute symptoms, late-onset acute symptoms, progressive general symptoms and permanent symptoms[23]. Some symptoms associated with metabolic disorders include lethargy, weight loss, jaundice and death[23].

2.1.3. Overview and prevalence of metabolic syndrome

Metabolic syndrome (MetS) is a cluster of risk factors including central obesity, dyslipidemia, hyperglycemia, and elevated blood pressure which increases the risk of cardiovascular disease and type 2 diabetes mellitus[2]. MetS, the epidemic of the 21st century, is a major public health threat, with approximately 1.4 billion people being affected by MetS[4]. There is a worldwide increase in the prevalence of MetS. For instance, Taiwan recorded an increase in the prevalence of MetS from 14% between 1993 - 1996 to 26% between 2005 - 2008[24]. Similarly, it has been reported that the prevalence of MetS in China increased from 14% in 2001 to 21% in 2009[25]. Several studies have revealed that the prevalence of MetS increases with age. For instance, a recent report indicated that the prevalence of MetS was approximately 18% among adults aged 20 to 39 whereas the prevalence of MetS was approximately 47% among adults aged 60 years or older[26]. A few studies have investigated the gender-related differences in the prevalence of MetS. It has

been reported that the prevalence of MetS of urban Chinese adults aged above 49 years was approximately 27% and 38% in men and women respectively[27]. Also, a population-based survey of cohort of subjects in India revealed that the prevalence of MetS in men and women aged 20 or above was approximately 25% and 13% respectively[28]. Furthermore, a recent report demonstrated significantly higher prevalence of MetS in women compared with men (35.6% vs 30.3%, respectively)[26]. These results suggest that age and gender might influence the prevalence of MetS in different ethnicities.

2.1.4. Definition and diagnosis of metabolic syndrome

The definition of MetS is complex. Generally, different associations define MetS based on certain cut-off points, although the defining risk factors of MetS are similar among the associations. Some associations with operating guidelines for MetS definition include World Health Organization (WHO), European Group for the Study of Insulin Resistance (EGIR), National Cholesterol Education Program - Third Adult Treatment Panel (NCEP: ATPIII), American Association for Clinical Endocrinology (AACE), International Diabetes Federation (IDF), American Diabetes Association (ADA) and American Heart Association (AHA). The definition of MetS according to various associations is presented in table 1.

Table 2.1 MetS definition and diagnosis

WHO	EGIR	NCEP:ATPIII	AACE	IDF
High insulin level	High fasting insulin concentrations – insulin resistance	*Any three of the following:*	Impaired glucose tolerance	Central obesity = WC (ethnicity and gender specific)
+ *Two of the following:*	+ *Two of the following:*		+ *Two of the following:*	+ *Two of the following:*
1. Abdominal obesity WC > 37″, BMI > 30 kgm^{-2}	**1.** WC ≥ 94 cm (M) ≥ 80 cm (F)	**1.** WC > 40″ (M) > 35″ (F)	**1.** Triglycerides ≥150 mg dL^{-1} HDL-C <40 mg dL^{-1} (M) <50 mg dL^{-1} (F)	**1.** Triglycerides ≥150 mg dL^{-1} HDL-C <40 mg dL^{-1} (M) <50 mg dL^{-1} (F)
2. Triglycerides >150 mg dL^{-1} HDL-C <35 mg dL^{-1} (M) <39 mg dL^{-1} (F)	**2.** Triglycerides >2 $mmolL^{-1}$ HDL-C <1 mg dL^{-1}	**2.** Triglycerides ≥150 mg dL^{-1} HDL-C <40 $mgdL^{-1}$ (M) <50 mg dL^{-1} (F)	**2.** BP in mmHg ≥ 130/85	**2.** BP in mmHg ≥ 130/85
3. BP in mmHg ≥ 140/90	**3.** BP in mmHg ≥ 140/90 or hypertensive medication	**3.** BP in mmHg ≥ 130/85		**3.** Fasting glucose ≥5.6 mmol L^{-1} or T2DM
4. Microalbuminuria >30 mgg^{-1}	**4.** Fasting glucose ≥6.1 mmol L^{-1}	**4**. Fasting glucose ≥110 mg dL^{-1}		

Criteria set out for the diagnosis of MetS according to a number of influential associations. AACE, American Association of Clinical Endocrinology; BMI, body mass index; BP, blood pressure; EGIR, European Group for the Study of Insulin Resistance; HDL-C, high-density lipoprotein cholesterol; IDF, International Diabetes Federation; MetS, metabolic syndrome; NCEP:ATPIII, National Cholesterol Education Program – Third Adult Treatment Panel; T2DM, type 2 diabetes mellitus; WC, waist circumference; WHO, World Health Organization; M, male; F, female (Modified from O'Neill and O'Driscoll[4]).

It is evident from Table 1 that the defining parameters of MetS are similar among the associations. Several attempts have been made to unify the definition of MetS. To this effect, a joint statement was published in 2009 by the IDF Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Health Federation; International Atherosclerosis Society; and International Association for the Study. According to the statement, the presence of three of the five MetS risk factors would suffice for diagnosis of MetS, with waist circumference requiring ethnic thresholds[29]. The definition of MetS is still under debate.

2.1.5. Metabolic syndrome risk factors

2.1.5.1. Central obesity

Obesity is defined as excess of body fat as measured by body mass index or waist circumference[30]. In addition to BMI and waist circumference, other parameters are also adopted in diagnosing central obesity including waist–hip ratio[31], waist-height ratio[32] and sagittal abdominal diameter[33]. Central obesity has been listed as the fundamental MetS risk factor by IDF since its guidelines for diagnosing MetS was updated in 2005[34]. Central obesity is strongly associated with different metabolic disorders including hypertension, insulin resistance and Type 2 diabetes Mellitus (T2DM)[7]. Central obesity has been shown to correlate more strongly with IR and other MetS risk factors compared to gynoid obesity[35].

The rising prevalence of central obesity is a major public health threat. As a result, the Scottish Intercollegiate Guidelines (SIGN) described obesity in 2010 as ‘a disease with

multiple organ-specific consequences'[6]. The US Centres for Medicare and Medicaid Services delisted obesity as a disease in 2004[36]. However, obesity has been recognized as a disease since 2013 by the American Medical Association (AMA)[7]. A recent report indicated that over 2 billion people, approximately 30% of the global population, were overweight or obese and 5% of global deaths were attributable to obesity[5]. It has also been projected that the rising incidence of obesity might affect half of the world's adult population by 2030[5]. The global financial burden of obesity was estimated to be $2 trillion or 2.8% of the global gross domestic product (GDP) in 2014[5], whereas the annual financial burden of childhood obesity has been reported to exceed $14bn in US alone[37]. The financial cost of obesity might have contributed to the recent identification of obesity as a disease by several countries.

Remedies for central obesity include routine exercise and healthy diet which are aimed at achieving a negative energy balance[38]. Drugs are prescribed for certain obese individuals, although the drugs are withdrawn if the side effects become intolerable. For instance, Sibutramine was initially prescribed for the management of central obesity but it has been withdrawn from world-wide markets due to reports of increased cardiovascular damage in some patients.

2.1.5.2. Hypertension

Hypertension, also known as high blood pressure, is a long term medical condition associated with persistently elevated blood pressure in the arteries[39]. Blood pressure is

defined by maximum and minimum pressures, respectively referred to as systolic and diastolic pressures. The systolic and diastolic blood pressures at rest range from 100 - 140 mmHg and 60 - 90 mmHg in adults respectively[40]. In adults, high blood pressure is often diagnosed as resting blood pressure persistently at or above 140/90 mmHg[41]. The adoption of lower blood pressure thresholds (135 mmHg systolic or 85 mmHg diastolic) for the diagnosis of MetS by several associations, including NCEP - ATP III, IDF and WHO might assist in early detection and prevention of future diseases such as stroke and heart attack. There are two main types of hypertension namely primary (essential) hypertension and secondary hypertension[41]. Primary hypertension accounts for approximately 90 - 95% of cases and is defined as high blood pressure resulting from unidentifiable lifestyle and genetic factors[41,42]. Some lifestyle factors that increase the risk of primary hypertension include excessive salt take, obesity, smoking and alcohol intake[41]. Secondary hypertension, on the other hand, accounts for the remaining 5 - 10% of cases and usually results from specific cause, such as chronic kidney disease, constriction of arteries or an endocrine disorder[41]. High blood pressure is closely linked to other MetS risk factors.

The prevalence of hypertension has been reported to vary between 16 and 37% of the global population[41]. The incidence of high blood pressure in children and adolescents has increased over the last three decades[43]. Although primary hypertension accounts for most cases of hypertension in children and adolescents, kidney disease has been identified as the most common secondary cause of hypertension in children and adolescents[44]. Recently, high blood pressure has been reported to play a significant role in approximately 18% premature deaths globally[45]. High blood pressure is the most common chronic medical problem

prompting visits to primary health care providers in US. The estimated costs of high blood pressure in US alone was $76.6 billion in 2010[46].

Although high blood pressure is a salient MetS risk factor, long term high blood pressure is known to accelerate the development of several diseases including cardiovascular diseases, vision loss and chronic kidney disease[47]. The prevention of other health complications is the goal of hypertension management which focuses on lifestyle modifications and use of drugs that can lower blood pressure[48]. Lifestyle modification for management of high blood pressure usually incorporates balanced diet, decreased salt intake and physical exercise to induce weight loss[41]. Pharmacological therapy is usually prescribed to supplement lifestyle changes in some hypertensive individuals[48].

2.1.5.3. Dyslipidaemia

Dyslipidaemia is characterized by abnormal amount of lipids including cholesterol and fats in the blood[4]. The defining features of dyslipidaemia include surge of fatty acids, apolipoprotein B (ApoB), high triglycerides, low HDL cholesterol and high small-density lipoprotein cholesterol (LDL-C)[49]. Dyslipidaemia is a clinical feature of MetS and is strongly associated with central obesity and insulin resistance[4].

In MetS subjects, dyslipidaemia is believed to result from the combination of excessive very-low-density lipoprotein, overproduction of ApoB, decreased breakdown of ApoB and increased catabolism of HDL cholesterol[49]. Overall, the three most common dyslipidaemia symptoms adopted in the routine diagnosis of MetS include fasting triglyceride-rich

lipoproteins, decreased HDL-C and increased LDL-C[50].

According to NCEP ATP-III guidelines, a balanced aerobic exercise program is recommended for subjects with elevated cholesterol level[51]. Cardiac rehabilitation and exercise training programs in subjects with coronary heart disease have been demonstrated to decrease triglycerides, total cholesterol and LDL-C by 15%, 5% and 3% respectively, but increase HDL-C by 6%[52]. In addition, exercise training in elderly patients with dyslipidaemia has been shown to elevate HDL-C by 15%[53]. Physical exercise is generally recommended as adjuvant therapy for elderly subjects with dyslipidemia[54].

2.1.5.4. Hyperglycemia

Hyperglycemia or high blood sugar refers to abnormally high level of circulatory glucose.

Hyperglycemia is commonly defined as fasting blood glucose level exceeding 126 mg/dl (7 mmol/Liter) or random blood glucose level greater than 200 mg/dL (11.1 mmol/Liter) measured in 2 or more occassions[55]. Diabetes mellitus, a serious illness that reduces life expectancy, is a consequence of hyperglycemia[56]. In fact, NCEP-ATP III recommended the use of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) as predictors of diabetes[51].

In an observational study involving 126 hospitals, the prevalence of hyperglycemia in intensive care unit subjects and non-intensive care unit subjects was 46% and 32% respectively[57]. Hyperglycemia was also diagnosed in approximately 38% of patients admitted to a US community teaching hospital[55]. Intriguingly, significantly higher in-

hospital mortality rate and increased length of hospital stay were reported in subjects with new hyperglycemia compared to subjects with prior history of diabetes and subjects with normoglycemia[55]. Hyperglycemia increase complications and mortality in subjects via osmotic diuresis resulting in hypovolemia, decreased glomerular filtration rate (GFR), impaired leukocyte functions, impaired collagen synthesis, poor wound healing and increased rate of hospital infections[58-60].

Hyperglycemia and diabetes are clinically managed by administration of exogenous insulin or by using drugs which increase insulin secretion, decrease glucose release from the liver, increase the use of glucose in the skeletal muscle and fat or delay the absorption of glucose from foods[56].

2.1.5.5. Insulin resistance

Insulin resistance (IR) was identified as the first MetS risk factor by Gerald Reaven in 1988[61]. IR is defined as a pathophysiological condition in which secreted insulin fails to elicit a normal insulin response in the peripheral target tissues including muscle, liver and adipose tissue[62]. IR is known to play crucial roles in the etiology of type 2 diabetes mellitus, hypertension and coronary artery disease[61]. Hence, syndrome X, now called MetS, was coined to describe the clustering of MetS risk factors with IR as the common factor[61]. The prevalence of IR in 2005 was estimated as 30 - 40% of the global population[63]. IR is related to several MetS risk factors including hyperglycemia, obesity, high triglycerides, low HDL cholesterol and hypertension[63].

In insulin-resistant individuals, insulin secretion by the β-cells is increased (i.e., hyperinsulinemia) to suppress hyperglycemia[62]. Although blood glucose remains abnormal in insulin-resistant individuals, hyperinsulinemia increases insulin actions in some sensitive tissues[62]. The imbalance between increase insulin actions and resistance to other actions of insulin has been suggested to contribute to the clinical manifestations of MetS[64]. In the long run, failure of the pancreatic beta cells to produce sufficient insulin to attenuate insulinemia leads to hyperglycemia and overt T2DM[65].

IR is generally managed via a combination of proper medication, healthy diet and exercise. Sedentary lifestyle has been identified as a risk factor of IR[66]. Hence, It has been reported that approximately 500 kcal/week increment in physical activity increases energy expenditure and reduces the lifetime risk of type 2 diabetes by 9%[67]. In a separate study, it was reported that vigorous exercise performed at least once a week reduced the risk of type 2 diabetes in women by 33%[68].

2.2. Muscle disorders

2.2.1. Overview of muscle

Muscle is a soft tissue which functions to produce force and motion. Muscle cells which are found in most animals contain protein filaments called actin and myosin[69]. Actin and myosin filaments are known to slide past one another, producing motion that alters the length and shape of the muscle[69]. In general, muscles control posture and locomotion and influence movement of internal organs, including the contraction of the heart and the movement of

food through the digestive system by peristalsis[70]. During embryogenesis, muscle tissues originate from the mesoderm in a process known as myogenesis[71]. During myogenesis, myoblasts differentiate and fuse into multi-nucleated fibers called myotubes[71]. Muscle hypertrophy refers to increase in muscle size via growth of the component cells[72], whereas muscle atrophy refers to decrease in muscle mass resulting from smaller number and size of the muscle cells[73]. Based on structure, there are three types of muscles namely skeletal or striated, cardiac, and smooth. Muscles are also classified as voluntary (cardiac and smooth) or involuntary (skeletal) muscles. Skeletal muscles in turn are usually divided into fast and slow twitch fibers[20].

2.2.2. Skeletal muscle

Skeletal muscle is a permanent, post-mitotic tissue with restricted turnover of cells[74]. Muscle activity involves several intracellular changes including increase in free intracellular Ca^{2+}, alteration in metabolites and hypoxia[74]. Although the concept of skeletal muscle fibre types is complex, muscle fibres are classified into various types based on histochemistry: I, IIa and IIb and IIc. Skeletal muscle is estimated to account for approximately 42% and 36% of adult male and female bodies respectively[75]. Skeletal muscles are histologically bundles of myofibrils which in turn are composed of sarcomeres. The sarcomeres are known to produce simultaneous contractions that shorten the muscle fiber, resulting in overall length change[76].

2.2.3. Types of skeletal muscle

In general, skeletal muscles are divided into 2 main types which are slow twitch and fast twitch muscles. Slow twitch muscles also known as Type I or red muscles are muscles with abundant capillaries, mitochondria and myoglobin, which give the muscles characteristic red color. Slow twitch muscles consume more oxygen via oxidation of fats or carbohydrates, and are known to sustain contractions for longer duration of time[77]. In contrast, fast twitch or Type II or white muscles have relatively fewer capillaries, mitochondria and myoglobin, which give the muscles pale color. Fast twitch fibers fatigue very rapidly but are known to produce quick and powerful anaerobic bursts of contractions[77]. Based on contractile speed and force generated, fast twitch muscles are divided into 3 major subtypes namely IIa, IIx and IIb[78].

2.2.4. Physiology of skeletal muscle contraction

Skeletal muscle accounts for approximately 40% of body weight and 25% of the basal metabolic rate[79]. As a result, skeletal muscle is a primary regulator of carbohydrate and lipid metabolism, being highly susceptible to glucose and fatty acid changes[80]. Metabolism of skeletal muscle is complex, depending on fiber type and level of stimulation[80]. The "twitch" which refers to action potential (AP) that causes contractile response is the basic unit of function of all skeletal muscle fiber types[77]. Changes in unbound calcium concentration is known to control signaling events between skeletal muscle excitation, contraction and relaxation. In brief, the processes involved in skeletal muscle function include increase in calcium concentration, binding of calcium to troponin which is a regulatory protein on the

actin filament, interaction of myosin with actin which generates the contractile response, decrease in calcium concentration, dissociation of calcium from troponin and decline of contractile activity or relaxation[77]. Abnormal skeletal muscle metabolism is often associated with increased fatty acid level which leads to accumulation of toxic lipid intermediates[80]. Skeletal muscle lipotoxicity is recognised as a primary cause of obesity and insulin resistance[80]. T2DM is also recognized as a disorder associated with alterations of carbohydrate, fat and protein metabolism primarily in the skeletal muscle[81].

2.2.5. Neuromuscular diseases

Neuromuscular diseases are diseases that affect muscles, nerves that control muscles and neuromuscular junctions[82]. Neuromuscular disease may result from autoimmune disorders, defective myelination of nerves, hereditary disorders, exposure to environmental chemicals or poisoning which includes heavy metal poisoning[82,83]. Neuromuscular diseases are numerous and include stroke, myasthenia gravis Parkinson's disease, multiple sclerosis, Huntington's disease and Creutzfeldt–Jakob disease. Neuromuscular diseases are known to cause muscle spasticity or paralysis depending on the location and the nature of disease. Symptoms of neuromuscular diseases include muscular weakness, rigidity, loss of muscular control, twitching, spasming, muscle pain[20].

2.2.6. Skeletal muscle disorders

The role of skeletal muscle in the pathogenesis of MetS is a subject of intense research. Skeletal muscle is widely studied in MetS as skeletal muscle solely accounts for

approximately one-quarter of the basal metabolic rate[79]. Several metabolic disorders are known to originate from skeletal muscle. For instance, it is believed that skeletal muscle insulin resistance occurs prior to hepatic insulin resistance[84]. Skeletal muscle, the largest insulin-sensitive tissue, is the principal tissue for insulin-stimulated glucose disposal[85]. As a result, abnormal skeletal muscle metabolism is often associated with MetS risk factors including central obesity, insulin resistance, hypertension, dyslipidaemia and hyperglycemia[85]. For instance, inactivation of insulin receptor gene in skeletal muscle of mice has been shown to increase plasma triglycerides and adiposity[86]. Also, a human study has demonstrated that insulin resistance in skeletal muscle promotes atherogenic dyslipidemia[84] which is a MetS risk factor.

Muscle atrophy is caused by natural aging process, diseases, inactivity or starvation[73]. Sarcopenia is age-associated degenerative decline of skeletal muscle mass and function[87]. Sedentary lifestyle is believed to constitute a significant risk factor of sarcopenia[88]. It also appears that sarcopenia is inevitable in aging adults since highly trained athletes have been reported to experience the effects of sarcopenia after the third decade of life[89,90]. There is currently no consensus therapy for the treatment of sarcopenia[87]. However, exercise has been recommended for management of sarcopenia based on a number of clinical and epidemiologic studies[91].

2.2.7. Pressure ulcer

Pressure ulcers or decubitus ulcers are damages to the skin, skeletal or bone tissues induced mainly by unrelieved mechanical pressure[13]. Pressure ulcers may result from extrinsic factors which include pressure, shear, friction, immobility, and moisture or intrinsic factors which include sepsis, local infection, decreased autonomic control, altered level of consciousness, increased age, vascular occlusive disease, anemia, malnutrition, sensory loss, and spasticity[92]. Although pressure ulcers greatly diminish quality of life, individuals with restricted movement are known to suffer more from the consequences of pressure ulcers. Pressure ulcers pose significant health, social and economic burden worldwide. It has been estimated that approximately 1 - 3 million people develop pressure ulcer, whereas approximately 60 000 people die from pressure ulcer complications in US each year[93,94].

2.2.8. Stages of pressure ulcer

It is generally difficult to track the transition of pressure ulcers from a simple stage to a complex one. The United States National Pressure Ulcer Advisory Panel (NPUAP) initially classified all pressure ulcers into four categories namely stage I (non - blanchable erythema), stage II (partial thickness loss), stage III (full thickness skin loss) and stage IV (full thickness tissue loss)[95]. Recently, two additional stages were added to the categories and these are the unstageable / unclassified pressure ulcer (displaying full thickness skin or tissue loss with unknown depth) and suspected deep tissue injury in which the depth of tissue loss is also

unknown. Another widely accepted classification of pressure ulcers is the placement of all types of ulcers into 2 categories namely superficial and deep pressure ulcers[96]. Deep pressure ulcers are clinically significant since the level of musculoskeletal damage is unknown, especially in dark-skinned individuals.

2.2.9. Pathogenesis of pressure ulcer

Although the pathogenesis of decubitus sores is complex and not completely known, it is believed that pressure applied on body surfaces occlude blood flow to the skin and underlying tissues, stimulating an array of degenerative events that ultimately result in death of the tissues. Removal of pressure on the compressed region leads to ischemia - reperfusion (I/R) injury in which a cascade of inflammatory activities accelerates cell death[97]. Some degradative pathways associated with pressure ulcer include oxidative stress, necrosis and apoptosis.

2.2.9.1. Oxidative stress

Oxidative stress refers to imbalance between production and detoxification of reactive oxygen species (ROS), which leads to cellular damage. Examples of reactive oxygen species generated during abnormal metabolism include superoxide radical, hydroxyl radical and hydrogen peroxide[98]. Occlusion of blood vessels as a result of unrelieved mechanical pressure induces hypoxia in the tissues. Hence, defective metabolism in pressure ulcers is hypothesized to produce free radicals that destroy all components of muscles including

proteins, lipids and deoxyribonucleic acid (DNA). Oxidative stress and DNA damage has been demonstrated in skeletal muscle subjected to moderate pressure[99].

2.2.9.2. Necrosis and necroptosis

Necrosis may be described as a form of unregulated digestion of cell components, leading to premature death of cells in living tissue[100]. On the other hand, necroptosis is described as regulated necrosis or a programmed form of necrosis cell death[101]. Necrosis often results from external stimuli to cells including infection, toxins or trauma. Necrosis and apoptosis were initially considered distinct processes. However, recent studies have revealed overlap between necrosis and apoptosis pathways[102]. In contrast to apoptosis, caspase activation and organized disposal of cellular contents into apoptotic bodies are absent in necrosis and necroptosis[103]. Also necrosis is known to be fatal whereas apoptosis is known to provide some beneficial effects[104]. Cellular death due to necrosis involves the activation of several receptors, loss of cell membrane integrity and unregulated discharge of cellular contents into the extracellular matrix[100]. Ischemia which causes depletion of nutrients and accumulation of toxic molecules is a classic example of a necrotic condition[100]. Hence, necrosis might complicate pressure ulcer by initiating inflammatory response, inducing microbial damage and creating collateral damage to surrounding tissues[105]. Oxidative stress and necrosis are suggested to be interrelated as Nitric oxide (NO) and reactive oxygen species (ROS) are stimuli of both degradative pathways[106].

2.2.9.3. Apoptosis

Apoptosis is a caspase-mediated form of programmed cell death[107]. In contrast to necrosis, apoptosis is viewed as a naturally regulated programmed and committed cause of cellular death. Caspases are known to execute apoptosis via distinguishable morphological features including exposure of phosphatidylserine on the outer end of the plasma membrane, chromatin condensation, DNA cleavage and cytoplasmic membrane blebbing[108]. Apoptosis proceeds via two main recognized pathways which are the extrinsic and intrinsic pathways. The extrinsic pathway is activated by distortion of cell-surface-expressed death receptors such as Fas receptor and tumour necrosis factor receptor, whereas the intrinsic pathway is activated by cellular stress and is regulated primarily at the level of mitochondria by the B cell leukemia/lymphoma-2 (Bcl-2) family of proteins[109].

In skeletal muscles, caspases are activated by internal and external stimuli and this depends on the balance between pro-and anti-apoptotic proteins of the Bcl-2 family, heat shock proteins, and inhibitors of apoptosis proteins[110]. Although different pathways such as oxidative stress, nitrosative stress, autophagy and inflammation are involved in pressure, apoptosis is the most frequently studied pathway in pressure ulcer diseases. Following 6 - hour long moderate compression in muscle, apoptosis has been demonstrated to be increased, as evidenced by increased apoptotic DNA fragmentation and caspases-dependent proteolytic events[111]. Although apoptosis was confirmed after the compression, apparent histopathological changes were absent, suggesting that molecular events at the cellular level precede apparent histopathological change[111]. As apoptotic events have been shown to precede histopathologic changes, molecules or factors which oppose or promote survival of

cells have stimulated research interests in pressure ulcer diseases in the last decade. For instance, resveratrol has been shown to attenuate apoptotic events in skeletal muscle under moderate compression, leading to protection against histopathologic damage[99].

2.2.10. Physiology of pressure ulcer healing

Pressure ulcers are generally believed to heal via the three major phases of wound healing which include inflammation, proliferation and maturation or remodeling[112]. The inflammatory phase involves constriction of injured vessels, destruction of injurious agents via recruitment of white blood cells, the proliferative phase involves matrix formation, angiogenesis and re-epithelialization, whereas the maturation phase involves collagen remodeling and possible scar formation[92]. The healing process of several pressure ulcers is often impeded by multiple factors despite complex processes aimed at ameliorating the ulcers[92]. As a result, pressure ulcers are often classified as chronic diseases.

2.2.11. Management of pressure ulcer

There is currently no cure for pressure ulcer. In clinical settings, pressure ulcers are managed via a combination of preventive control measures including stimulation of physiologic wound healing with pressure relief, debridement, control of wound infection, surgical treatment and nutrition supplementation[92]. Prevention of pressure ulcers has been recommended for the optimal management of pressure ulcer, even after successful treatment of previous ulcers.

2.2.12. Current trends in the management of pressure ulcer

The discovery of animal based models of pressure ulcer in the past two decades has deepened existing knowledge and highlighted several exciting new treatment possibilities for pressure ulcers. For instance, pharmacological inhibitions of caspases and proteasome which are degradative proteins have been shown to be beneficial in rat model of pressure ulcer[111,113]. Resveratrol which is a natural phenolic ingredient abundant in red wine has been recently demonstrated to mitigate oxidative DNA damage, down-regulate oxidative stress and apoptosis and attenuate histological damage in rat model of pressure ulcer disease[99]. In a separate investigation, resveratrol was reported to mitigate the protein abundances of apoptotic and catabolic proteins including p53, Bax, Muscle atrophy F-box (MAFbx) and ubiquitin and attenuate the increase in enzymatic activities of caspase 3 and proteasome induced by compression injury to rat skeletal muscle[14]. Ultimately, the entire molecular pathways associated with pressure ulcer remain elusive and more research is needed to identify signalling molecules that might effectively halt degradative pathways associated with pressure ulcers.

2.2.13. Role of sirtuins in pressure ulcer

Recently, sirtuins have attracted attention from researchers, due to their influence on wide variety of cellular processes including metabolism, aging, transcription, apoptosis, inflammation and stress resistance[114,115]. Sirtuins are proteins that possess ribosyltransferase

or deacylase activity including deacetylase and desuccinylase activities[115]. Sirtuins have been proposed as therapeutic targets for adult diseases such as type II diabetes mellitus[116]. Although seven sirtuins have been described in humans, SIRT1 is the most widely studied member of the sirtuin family[117]. SIRT1 is known to influence several important biological processes including energy expenditure, glucose and insulin metabolisms, adipose tissue inflammation, hypoxia and endoplasmic reticulum stress[118,119]. Resveratrol, a SIRT1 activator, has been suggested to extend lifespan[120]. Recent findings regarding the role of SIRT1 in ischemia reperfusion injury suggest that SIRT1 pathway might represent potential therapeutic target for the management of pressure ulcers. SIRT1 deacetylates pro-apoptotic proteins such as tumor-suppressor p53 and anti-apoptotic proteins such as Ku70, resulting in inhibition of downstream apoptotic signaling events[121,122]. SIRT1 is also known to initiate hypoxic stress response via deacetylation of hypoxia inducible factor 2 alpha (HIF2α)[123]. Recently, the anti-apoptotic and anti-catabolic effects of resveratrol on compression injury in skeletal muscle have been demonstrated to be mediated via the action of SIRT1[14]. In particular, resveratrol was demonstrated to prevent the pathohistological damages induced by moderate compression, concomitant with increase expression and activity of SIRT1[14]. Therefore, it is probable that activators of SIRT1 might be clinically relevant in pressure ulcer disease.

Exercise

Role of exercise in the management of metabolic disorders

Several observational studies conducted over 4 decades ago suggested that morbidity and mortality due to vascular disease were inversely related to physical exercise. For example, Ford and DeStefano[124] reported that sedentary lifestyle was significantly associated with higher rates of coronary death in a sample of 492 diabetic men and women from the National Health and Nutrition Examination Survey (NHANES). Also, follow-up studies of over 8,000 men in a preventive medicine clinic in US revealed a higher risk of mortality for physically inactive men compared to physically active men[125].

The protective role of exercise in metabolic disorders has been attributed to favorable effects of exercise on cardiovascular risk factors and a direct action of exercise on the heart, leading to increased myocardial oxygen supply, decreased myocardial oxygen demands, enhanced collateral coronary circulation, enhanced myocardial contraction and electrical stability of the heart[126]. In addition, chronic exercise has been shown to promote a reduction in body fat[127], leading to decrease in the degree of central obesity. Furthermore, exercise has been shown to modulate energy homeostasis via enhancing mitochondrial function, enhancing production of brain-derived neurotrophic factor (BDNF), inducing autophagy and promoting antioxidant system[128].

Overview of Yoga

Yoga refers to systems or practices derived from ancient Indian traditions. Yoga essentially involves simple to complex body postures (asanas) maintained for a period of time, aimed at improving breath control, voluntary meditation, physical strength and balance[129]. There are many schools or systems of yoga in different parts of the globe. Many aspects of yoga incorporate different types of exercises ranging from gentle stretching exercises to complex postural acts. Yoga exercise is known to relieve stress and improve musculoskeletal strength[9]. In addition, yoga exercises are believed to benefit the circulatory system and increase flexibility among adherents[9]. Although yoga originated in Asia, yoga has enjoyed a tremendous growth in popularity as an adjunct to healthy living since it was introduced in the west about a century ago.

Brief history of Yoga

Yoga as a tradition was believed to have originated in the Indian peninsula approximately 5000 years ago. Yoga rudiments on the other hand are believed to have emerged from the Indus valley region which is current day Pakistan[129]. Yoga Sutra is believed to represent the earliest recorded history of yoga tradition. Yoga Sutra text dating back to the period between 200 BC and 300 AD was asserted to have been written by historically renowned yoga teacher and Hindu philosopher named Patanjali[129]. Yoga was introduced in Europe and America by Swami Vivekananda who popularized Eastern Hindu philosophy in the late nineteenth and

early twentieth centuries[129]. The United Nations General Assembly approved a resolution in 2014 establishing 21 June as "International Day of Yoga".

Hatha Yoga

Among the various forms of yoga that exist, Hatha yoga is the most commonly practiced. Hatha yoga focuses on physical exercises aimed at stretching and stimulating the spine and muscles in coordination with breath control[11]. Although the mechanism of action of yoga is not entirely known, Hatha yoga is believed to stabilize the hypothalamic-pituitary-adrenal axis and sympathoadrenal activity[11].

Beneficial effect of Yoga exercise

Comparisons of yoga exercises and traditional aerobic exercises based on cardiometabolic risk factors revealed no significant difference, suggesting similar effectiveness of yoga exercise and aerobic exercises and possibly similar mechanisms[11]. A recent systematic review reported that yoga was effective for low back pain in the short-term and in the long-term[130].Yoga exercises have been reported to decrease anxiety, depression and blood pressure in subjects with cardiac diseases[131]. Recommendations have also been proposed for the ancillary use of yoga for subjects with various heart diseases including coronary heart disease, heart failure and cardiac dysrhythmia[10]. A review of 11 trials (800 participants) conducted by the Cochrane Collaboration maintained that yoga has favourable effects on diastolic blood pressure, HDL-C and triglycerides[132]. In addition, a recent study suggested

that yoga exercises might be beneficial in managing cardiometabolic risk factors[11]. In particular, individuals who participated in yoga exercises reported significant decreases in body weight, diastolic blood pressure, total cholesterol, triglycerides and heart rate compared to non-exercise controls[11]. In a separate study, yoga exercise was demonstrated to improve MetS risk factors including systolic and diastolic blood pressure, waist circumference, waist/hip ratio, total cholesterol, HDL-C, very low density lipoprotein and insulin resistance[133]. Therefore, yoga exercise might represent a useful therapeutic intervention for managing metabolic disorders.

Combined yoga and Ayurveda therapy in subjects with Duchenne muscular dystrophy (DMD) has been reported to yield positive outcome in mobility, self-care, and respiratory ease at the end of 18 months[134]. A pilot study supports the potential benefits of yoga for treatment of fibromalaysia in women[135]. In the study, women with fibromalaysia assigned to a yoga program reported significantly greater improvements on standardized measures of fibromalaysia symptoms and functioning including pain, fatigue and mood in comparison to women with fibromalaysia who received no yoga intervention[135]. The beneficial effect of yoga in managing fibromalaysia was supported by Curtis et al. who demonstrated that women with fibromalaysia who participated in 8-week yoga program achieved decreased pain and cortisol level but improved psychological functioning and mindfulness in comparison to controls without yoga intervention[136].

2.3. Ghrelin axis

2.3.1. Brief history and overview of ghrelin gene products

There are three ghrelin gene products namely unacylated ghrelin (UnAG), acylated ghrelin (AG) and obestatin[137]. The discovery of ghrelin gene products was preceded by pharmacological and molecular characterization of growth hormone secretagogues (GHSs) in 1996[138]. AG and UnAG were discovered by reverse pharmacology in 1999[139], whereas obestatin was discovered in 2005 by comparative genomic analyses[137]. Ghrelin O-acyltransferase (GOAT), the enzyme that produces AG via octanoylation, was identified and characterized in 2008 by two independent research groups[140,141]. The post-translational modification of AG at serine 3 is known to be indispensable for the actions of AG[142]. In humans, fasting total ghrelin and obestatin levels have been shown to correlate negatively with body mass body mass index[143-145] and body fat[144,146]. AG is known to increase meal size[147,148] and induce the secretion of GH[139], leading to adiposity[149] in rats and humans. In contrast, UnAG lacks octanoylation at serine 3 but remains the major form of circulatory ghrelin[150]. Contrary to earlier reports that UnAG is an inert molecule, UnAG has been demonstrated to regulate feeding[148,151] control gut motility[148,152], influence adipogenesis and body size development[148,153] and participate in in vitro cell proliferation and survival[154,155]. Hence, UnAG is not merely an inert molecule but might be essential in the regulation of different metabolic pathways. Although obestatin was initially suggested to inhibit food intake, the effects of obestatin on feeding remain controversial[137].

2.3.2. Tissue expression of ghrelin gene products

The X/A cells of gastric oxyntic gland have been reported as cells with the highest concentration of AG[139]. AG has also been detected in the colon, hypothalamus, pituitary gland, pancreas, adipose tissue, heart and lung[139,156,157]. Using immunofluorescence double staining, it has been demonstrated that AG and UnAG-positive reactions overlapped in closed-type round cells, whereas UnAG-positive reaction was found in open-type cells in which AG was negative[158]. It was also shown that both AG and UnAG-positive closed-type cells contained obestatin whereas UnAG-positive open-type cells contained somatostatin[158]. UnAG has been demonstrated to be released from perfused rat stomach at pH 2 whereas AG release was not affected by intragastric pH[158]. Obestatin has been mapped in the gastrointestinal tract, within the A-like cells and oxyntic glands of the gastric mucosa, cholinergic neurons and in the testis[159].

2.3.3. Ghrelin O-acyltransferase

Ghrelin O-acyltransferase (GOAT) is a polytopic membrane-bound enzyme that activates AG via attachment of octanoate to serine-3 of ghrelin[140,141]. GOAT belongs to the 16-member family of membrane-bound O-acyltransferases[160]. GOAT is the only enzyme with extensive expression similar to ghrelin distribution[160]. The mechanistic explanation of increased AG during fasting is supported by research showing that chronic undernutrition increased the expression of GOAT mRNA levels in stomach[161].

2.3.4. Growth Hormone Secretagogue Receptor

AG receptor, Growth hormone secretagogue receptor (GHSR), is a G protein-coupled receptor[162] which regulates energy homeostasis and body weight[163]. GHSR gene encodes GHSR1a which is pharmacologically active and GHSR 1b which is pharmacologically inactive[138]. The expression of GHSR1a is mainly restricted to brain centers associated with control of energy homeostasis including the hypothalamic nuclei, area postrema, nucleus of the solitary tract, dorsal motor nucleus of the vagus, hippocampus, dopaminergic neurons in the ventral tegmental area and substantia nigra, parasympathetic preganglionic neurons, dorsal and medial raphe nuclei, pituitary and the dentate gyrus[164,165]. Besides the central nervous system, GHSR1a is also distributed in peripheral tissues including cardiac and pulmonary tissues, liver, kidney, pancreas, stomach, colon, adipose tissue and immune cells[164,166]. Although GHSR1b is physiologically inactive, the distribution of GHSR1b in all tissues is more extensive compared to GHSR1a[164]. During fasting, increased GHSR1a mRNA was observed in the arcuate nucleus of obese Zucker rats, coincident with high level of plasma AG[167]. This suggests that increased and decreased AG and GHSR 1a might be simultaneously stimulated by hunger and satiety signals respectively. The binding of AG to GHSR1a activates several downstream pathways including mitogen-activated protein kinase, Protein kinase B (Akt), nitric oxide synthase and AMP-activated protein kinase (AMPK) pathways in different systems[163]. Besides AG, several synthetic peptide and non-peptide ligands including hexarelin and JMV2951 have been shown to bind GHSR 1a, leading to the release of GH[168].

2.3.5 Concentration and catabolism of ghrelin gene products

Plasma UnAG/AG ratio differs according to research, ranging from 3:1[169] to 9:1[150,170]. Plasma UnAG ranges from 97.2 fmol/mL - 35.5 pg/mL whereas AG ranges from 11.1 fmol/mL - 4.6 pg/mL[171,172]. The range of plasma obestatin is 68 - 267 pg/mL[173,174]. The half-life of UnAG and AG has been estimated as 27 - 31 min and 9 - 13 min respectively[175], whereas the half-life of obestatin in plasma, liver and kidney has been estimated as 42 min, 13 min and 138 min respectively[176]. Ghrelin is degraded by blood esterases including platelet activating factor [67] acetylhydrolase and butyrylcholinesterase[177-179], whereas obestatin is degraded by proteases including aminopeptidase and post-prolyl endopeptidase which abound in blood, liver and kidney[176].

2.3.5. Unacylated ghrelin

2.3.5.1. Effect on feeding

It is currently believed that UnAG influences feeding patterns in different animal species. UnAG has been demonstrated to be increased by hunger but suppressed by feeding in mice[180], rats[181], and humans[182]. Conversely, centrally and peripherally administered UnAG has been reported to decrease food intake in mice[152] and rats[151,183]. Furthermore, central administration of UnAG has been demonstrated to increase food intake during the light phase in mice and rats[184]. UnAG has also been reported to inhibit dark-phase cumulative food intake and body weight gain in freely fed obese Zucker rats[185]. On the other hand,

Neary et al.[186] showed that UnAG administration did not affect feeding in fasted and freely fed mice.

2.3.5.2. Effect on glucose metabolism

Several groups have investigated the role of UnAG in glucose metabolism. In particular, UnAG has been demonstrated to oppose the effect of AG on glucose metabolism. For instance, the decrease in insulin level and increase in plasma glucose induced by AG were reversed when AG was co-administrated with UnAG in healthy humans[187]. The beneficial effect of UnAG in glucose regulation was substantiated by a studying showing that co-administration of UnAG and AG attenuated the rise in glucose and insulin levels induced by AG in adult-onset GH-deficient patients[188]. Furthermore, intravenous administration of UAG has been recently shown to enhance glucose tolerance, increase postprandial insulin secretion and decrease free fatty acid levels in healthy humans compared to saline-infused controls[189]. Also, continuous overnight infusion of UnAG has been demonstrated to improve glycemic control in obese subjects with type 2 diabetes via the suppression of AG levels[190]. Transgenic mice overexpressing UnAG have been reported to record lower blood glucose and insulin levels and demonstrated greater hypoglycemic response to insulin infusion[191,192]. UnAG and its analog, AZP531, have been shown to prevent short-term glucose intolerance and insulin resistance induced by high fat diet[193]. The antilipolytic effect of UnAG and synthetic GHS has been suggested to be mediated through a receptor distinct from GHSR 1a[194]. Hence, the hypoglycemic properties of UnAG underscore the possible beneficial effect in the treatment of disorders resulting from abnormal glucose and insulin levels.

2.3.5.2. Effect on lipid metabolism

Using microarrays, genome-wide expression pattern of UnAG in fat, muscle and liver of GHSR-deficient mice has been recently investigated[195]. UnAG was reported to regulate clusters of genes involved in glucose and lipid metabolism and enhanced insulin sensitivity in fat, muscle and liver of GHSR-deficient mice[195]. Previous studies have focused on relationship between total ghrelin or AG and lipid metabolism, as UnAG was considered inactive. For example, total ghrelin has been shown to influence lipid metabolism in skeletal muscle, liver and adipose tissue[196]. Also, positive association between plasma total ghrelin and HDL-C has been demonstrated[197]. A study has reported inverse correlations between UAG and triglycerides and LDL-C and positive correlation between UAG and HDL-C in humans[198]. In addition, exercise was shown to increase UnAG, concomitant with reduced level of total cholesterol and triglycerides[198].

2.3.5.4. Effect on cardiovascular system

Microinjection of UnAG in the nucleus tractus solitarius has been demonstrated to decrease blood pressure in rats[199]. In rats, UnAG has been shown to prevent doxorubicin-induced myocardial fibrosis and apoptosis[200]. UnAG has been demonstrated to improve cardiovascular risk prediction in hypertensive individuals[201]. Hence, UnAG has been proposed as a useful cardiometabolic marker for predicting atherosclerosis in hypertensive subjects[202]. Recently, the beneficial effect of UnAG on worksite blood pressure in obese subjects has been investigated. The results revealed significant inverse correlations of

UnAG with resting systolic blood pressure (SBP) and diastolic blood pressure (DBP), 24 - hour SBP and DBP, worksite SBP, home SBP and sleep DBP[203]. In addition, negative associations were reported between UnAG and blood pressure changes from sleep to morning, sleep to work, and sleep to home[203]. These results suggest that UnAG might play beneficial roles in blood pressure regulation in obese condition.

2.3.6. Acylated ghrelin

2.3.6.1. Effect on feeding

Among the different molecules secreted by the gastrointestinal tract in response to food intake, AG remains the only peptide that stimulates appetite and increases meal size. As a result, AG is known to function as a signal for hunger and meal initiation[147,148]. This is supported by studies showing that fasting induced increased secretion of AG whereas feeding inhibited the secretion of AG in mice and rats[180,204]. Similarly, preprandial rise and postprandial fall in AG have been demonstrated in humans[205,206]. Central administration of AG has been reported to promote fat ingestion in rats[207], whereas long-term or repeated injection of AG has been demonstrated to stimulate appetite in humans with chronic wasting diseases, such as chronic obstructive pulmonary and chronic kidney diseases[208,209]. A decade ago, a novel organ-culture model of gastric tissue explants obtained from rat donors was validated for ex vivo experiments[210]. It was demonstrated that fasting induced mRNA expression and gastric release of AG whereas the mRNA expression and gastric release of AG were reverted by 15 min of refeeding, prior to stomach extraction[210]. Taken together,

these studies support the physiological role of AG in meal initiation and feeding.

2.3.6.2. Effect on water intake

AG is known to participate in the regulation of body fluid homeostasis. It has been demonstrated that centrally and peripherally administered AG inhibited water intake in rats[211]. Subsequent research also revealed that centrally administered AG strongly inhibited water intake induced by angiotensin II and hypovolemia in rats[212]. The role of AG in water regulation was further confirmed by a studying showing that AG attenuated saline intake stimulated by angiotensin II, by water deprivation followed by partial rehydration, and by dietary sodium deficiency[213].

2.3.6.3. Effect on glucose metabolism

Ghrelin has been shown to control glucose homeostasis by regulating pancreatic Ucp2 expression and insulin sensitivity[214]. It was demonstrated that ghrelin gene deletion in mice augmented glucose dependent insulin secretion from the pancreatic β cell by reducing Ucp2 expression[214]. Exogenous AG infusion has been shown to decrease insulin but increase glucose levels in human volunteers[215,216]. Also, intravenous infusion of AG has been reported to suppress insulin concentration but induce hyperglycemia in human volunteers[217]. The in vivo effect of AG on insulin secretion was subsequently confirmed in rodents. AG was shown to inhibit glucose-stimulated release of insulin when infused into the portal vein, whereas AG infusion into the femoral vein failed to inhibit insulin secretion[218]. Furthermore, hepatic vagotomy or co-infusion of AG with atropine methyl bromide decreased the inhibitory effect of ghrelin on glucose-stimulated insulin secretion[218].

2.3.6.4. Effect on lipid metabolism

AG has been shown to inhibit isoproterenol-induced lipolysis in rat adipocytes via a receptor distinct from GHSR 1a[194]. Subcutaneous injection of AG in rats has been demonstrated to stimulate mitochondrial and lipid metabolism genes, thereby favoring triglyceride deposition in liver and skeletal muscle[219].

2.3.6.5. Effect on cardiovascular system

The first indication that AG plays a role in the cardiovascular system was reported in 2001 by Nagaya et al[220]. Short-term intravenous infusion of AG was shown to decrease mean arterial pressure, increase cardiac index, stroke volume index and epinephrine secretion, without alteration of heart rate in healthy subjects[220]. Short-term intra-arterial infusion of AG was subsequently shown to increase forearm blood flow in healthy volunteers in a dose-dependent manner[221]. In rats, microinjection of AG in the nucleus tractus solitarius was reported to significantly decrease mean arterial pressure and heart rate and suppressed renal sympathetic nerve activity[222]. However, prior intravenous injection of pentolinium, a ganglion-blocking agent, was shown to abolish the cardiovascular responses elicited by microinjection of AG into the nucleus tractus solitarius[222].

2.3.6.6. Regulation of energy homeostasis

In addition to stimulating appetite and eating, AG influences body weight and adiposity. It has been reported that long-term or repeated AG infusion induced weight gain in rats[223] and in humans with chronic wasting conditions, including chronic heart failure and chronic obstructive pulmonary disease[208,224]. The ability of AG to enhance fat ingestion in rats has

been suggested to contribute to the development of obesity[207]. The administration of AG has been suggested to decrease energy expenditure in mice and rats and induce adipogenesis via several mechanisms including the up-regulation of messenger ribonucleic acid (mRNA) expression of uncoupling protein 1 (UCP1) in brown fat tissue and uncoupling protein 2 (UCP2) in white adipose tissue[225], increase in respiratory quotient[226] and decrease in oxygen consumption[227].

2.3.6.7. Protection of gastric mucosa

Existing evidence suggest that AG might protect the gastric mucosa. The first evidence for this proposal was deduced from experiment demonstrating that intracerebroventricular AG infusion in rats decreased ethanol-induced gastric ulcers in a dose-dependent fashion[228]. Subsequent studies indicated that intraperitoneal AG pretreatment prevented ethanol-induced, stress-induced or ischemia-reperfusion-induced gastric mucosal lesions[229,230]. The protective effect of AG on ethanol-induced, stress-induced, or ischemia-reperfusion-induced gastric mucosa damage were attributed to increase in gastric mucosa blood flow, generation of mucosal prostaglandin E2 or activity of sensory nerves[229,230]. In addition, AG has been demonstrated to modulate cell proliferation, thereby promoting the regenerative potential of the gastrointestinal epithelium in doxorubicin-treated mice[231].

2.3.6.8. Control of secretion

Some nutrient-regulating peptides including cholecystokinin (CCK) and somatostatin (SST) have been shown to affect AG secretion[232]. Although the factors and mechanisms that regulate AG response to nutrient fluctuation remain largely unknown, it has been suggested that stomach AG secretion is modulated by both the cholinergic and adrenergic branches of

the autonomic nervous system[233]. In particular, vagotomy was demonstrated to acutely inhibit AG secretion, but favored AG release from the stomach at later time points[233]. Furthermore, stomach AG mRNA level was found to be significantly upregulated in vagotomized rats but remained unchanged after fasting[170]. Earlier investigations showed that circulating AG levels in humans are increased by cholinergic agonists but reduced by cholinergic antagonists[234].

2.3.7. Obestatin

Effect on feeding

Although obestatin was initially identified as an anorexic peptide[137], the effects of obestatin on feeding and weight control is a subject of debate[17]. For instance, Zhang et al.[137] demonstrated that obestatin inhibited food intake in rats whereas Gourcerol et al.[235] showed obestatin administration failed to modify food intake in rats. A reduction in the number of obestatin-positive cells has been reported in the gastric mucosa of overweight or obese subjects with central obesity[236,237]. Obestatin has also been reported to decrease in response to insulin administration in normoglycaemic rats[238]. The contrasting results indicate that obestatin might be regulated in a complex manner and calls for in-depth exploration of mechanisms regulating obestatin actions.

Effect on glucose metabolism

In a series of experiments, Ren et al. demonstrated that obestatin alone or in combination with glucose inhibited insulin secretion in rats in a dose-dependent fashion[239]. In a separate investigation, exogenous obestatin was shown to inhibit insulin release from isolated rodent

pancreatic islets[240]. Contrary to reports that obestatin inhibited insulin secretion, other researchers have reported that intravenous obestatin affected neither basal nor intravenous glucose-stimulated insulin secretion and glucose homeostasis in fasted rats[241,242]. The role of obestatin on insulin and glucose homeostasis might be related to the concentration of obestatin as demonstrated in a recent study. Obestatin was shown to potentiate insulin response to glucose at a low concentration, but obetatin inhibited insulin release in response to glucose stimulation[243]. These modulating effect of obestatin on glucose and insulin metabolism suggest that obestatin alteration might contribute to hyperglycemia or insulin resistance.

Effect on cardiovascular system

Obestatin levels has been demonstrated to be significantly increased in congestive heart failure subjects, with or without cachexia compared to healthy controls[244]. Circulatory obestatin has also been reported to be increased in spontaneously hypertensive rats, suggesting that obestatin might play a role in the control of blood pressure[245]. In vitro and in vivo studies have suggested that obestatin might protect rat cardiomyocyte from I/R injury via anti-inflammatory, anti-cytotoxic and anti-apoptosis mechanisms[246].

2.3.8. Insulin

2.3.8.1. Overview

Insulin is a metabolic peptide hormone produced by β-cells of the pancreas. The structure of insulin varies slightly between animal species. In particular, human insulin has a

molecular mass of 5808 Da and is composed of 51 amino acids. Human insulin contains two chains linked together by disulfide bonds[247].

2.3.8.2. Metabolism and regulation

The primary stimulus of insulin release from the β-cells of the pancreas is blood glucose concentration. Pancreatic β-cells secrete insulin into the blood in response to high blood glucose concentration. On the other hand, secretion of insulin from the pancreas is inhibited when blood glucose concentration is low[248]. Glucose level in the extracellular fluid is believed to be maintained primarily by insulin secretion within very narrow limits at rest, after meals, during exercise and starvation[248].

The release of insulin from the β-cells is preceded by entry of glucose into the β-cells. Glucose is known to enter the β-cells through low-affinity glucose transporters called GLUT2[249]. The glucose that enters the β-cells is phosphorylated to glucose-6-phosphate (G-6-P) by glucokinase (hexokinase IV)[249]. Glucose-6-phosphate is subsequently metabolized in the glycolytic pathway and Krebs cycle, leading to the production of ATP molecules[250]. ATP produced in the β cell significantly increases the amount of calcium ions in the cytoplasm, resulting in the release of insulin stored in intracellular secretory vesicles into the blood[18]. Besides blood glucose concentration, other known stimuli of insulin release include arginine and leucine, parasympathetic release of acetylcholine, sulfonylurea, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP)[251]. Between meals, blood insulin level ranges between 8 – 11 μIU/mL (57–79 pmol/L)[252].

It has been reported that endogenously synthesized insulin is degraded within approximately 1 hour after its release from the β-cell, whereas the half-life of insulin is approximately 4 – 6 minutes)[253]. After binding to its receptor, insulin is either degraded by the cell or released back into the extracellular fluid (ECF). The degradation of insulin occurs mainly in the liver and the kidneys: the liver degrades most insulin during first-pass transit, whereas the kidney clears a major portion of circulatory insulin[18]. Degradation of insulin is known to begin with endocytosis of the insulin-receptor complex, followed by the action of insulin-degrading enzyme[253].

The destruction of pancreatic β-cells by autoimmune reactions decreases the amount of insulin synthesized and secreted into the blood, leading to type 1 diabetes mellitus (T1DM)[254]. T2DM which develops mainly in adulthood is often related to tissue insulin resistance and relative insulin deficiency[254]. Insulin injections are usually given to diabetics to maintain blood glucose level within normal bounds. The effectiveness of insulin injection in humans is limited by the slight structural variations of insulin from other species. Prior to the production of large quantities of human insulin by recombinant DNA technologies, porcine insulin was widely adopted in the treatment of type 1 diabetics as a result of the close structural similarities of human and bovine insulin[255].

2.3.8.3. Physiologic effects

In general, insulin is known to affect virtually all aspects of metabolism. Insulin regulates the metabolism of major dietary substrates including carbohydrates, fats and proteins[18]. The principal action of insulin is regulation of blood glucose concentration. Insulin promotes the absorption of glucose into the liver, skeletal muscle and adipose tissues. In the liver, skeletal

muscle and adipose tissues, the absorbed glucose is converted into glycogen and fats via glycogenesis and lipogenesis respectively[18]. In several tissues including muscle and adipose tissues, insulin promotes the insertion of glucose transporters called GLUT in the cell membranes, thereby promoting uptake of glucose and decreasing blood glucose concentration[18]. Also, insulin stimulates hexokinase and phosphofructokinase enzymes which catalyze glycogenesis when glucose levels are high and simultaneously decrease glycogenolysis by inhibiting the enzyme glucose-6-phosphatase[18]. Insulin is also known to increase DNA replication and protein synthesis via increase of amino acid uptake, increase esterification of fatty acids and lipid synthesis, enhance learning and memory[256], enhance fertility by stimulating release of gonadotropin-releasing hormone (GnRH) from the hypothalamus[257], decrease autophagy[258] and decrease renal sodium excretion[259].

2.3.9. Growth hormone

2.3.9.1. Overview

Growth hormone, also known as somatotropin, is a 191-amino acid, single-chain polypeptide hormone that stimulates growth and cell reproduction[260]. The molecular weight of growth hormone is 22,124 daltons[261]. Growth hormone is synthesized, stored, and secreted by somatotropic cells of the anterior pituitary gland.

2.3.9.2. Metabolism and regulation

Secretion of growth hormone is regulated by the hypothalamus. GH release by the pituitary is regulated by balance between growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH) released by the hypothalamus into the hypophyseal

portal venous blood surrounding the pituitary. GHRH and GHIH in turn are affected by physiological stimuli of GH secretion including exercise, nutrition, sleep and free fatty acids[262]. Apart from GHRH and GHIH, other factors which affect GH secretion include other hormones, age, gender, nutrition, physical exercise and stress[263].

GH is synthesized and secreted in a pulsatile manner in response to hypothalamic stimuli. It has been reported that GH secretion varies widely between days and individuals. Although approximately 50% of GH secretion occurs during the third and fourth non-rapid eye movement (NREM) sleep stages[264], it has been reported that GH secretion is highest approximately 1 hour after onset of sleep, with plasma level ranging from 13 to 72 ng/mL[265].

2.3.9.3. Physiologic effects

Growth hormone is generally considered an anabolic molecule that promotes growth in virtually all tissues of the body. Growth hormone increases height during childhood by at two principal mechanisms. Firstly, growth hormone is known to directly stimulate division and multiplication of chondrocytes of cartilage via activation of the Ras-mitogen-activated protein kinase/Extracellular signal-regulated kinase (MAPK/ERK) pathway[266]. Secondly, through the Janus kinase - signal transducers and activators of transcription (JAK - STAT) signaling pathway, growth hormone stimulates the production of insulin-like growth factor 1 (IGF-1), which in turn enhances growth in tissues[266]. The liver is the principal organ that produces IGF-1 in response to GH input, although additional IGF-1 is generated within target tissues. The release of growth hormone elevates the concentration of glucose and free fatty acids[260]. Growth hormone is known to contribute to the regulation of blood glucose by decreasing liver uptake of glucose and promoting gluconeogenesis in the liver[261]. Evidence

exists that growth hormone directly promotes fetal myocardial growth and function during embrogenesis[267]. In particular, it was demonstrated that growth hormone induced mRNA expression for specific contractile proteins and increased the force of contraction of mouse myosin during embryogenesis[267].

2.3.10. Insulin-like growth factor-1

2.3.10.1. Overview

Insulin-like growth factor 1 (IGF-1), also called somatomedin C, is a protein that in humans, is encoded by the IGF1 gene, with a molecular structure similar to insulin[268,269]. IGF-1 is a peptide which plays important roles from childhood to adulthood. IGF-1 consists of 70 amino acids in a single chain linked by three intramolecular disulfide bridges and it has a molecular weight of 7,649 Dalton[270].

2.3.10.2. Metabolism and regulation

IGF-1 is produced mainly by the liver as an endocrine hormone. IGF-1 is also produced in target tissues where it acts in a paracrine/autocrine fashion[271]. The release of IGF-1 is stimulated by growth hormone (GH) but inhibited by undernutrition, dysfunctional growth hormone receptors and failures of the downstream signaling pathways. More than 80% of IGF-1 is transported bound to plasma proteins called Insulin-like growth factor-binding proteins (IGF-BP)[271].

2.3.10.3. Physiological effects

IGF-1 is known to mediate the effects of growth hormone[266]. Growth hormone is synthesized in the anterior pituitary gland and stimulates the liver to produce IGF-1. IGF-1

then stimulates systemic body growth in almost every cell in the body including skeletal muscle, cartilage, bone, liver, kidney, nerves and skin. IGF-1 has also been shown to regulate DNA synthesis, cell growth and development[272]. IGF-1 analogs are used in clinical practice to stimulate growth. For example, Mecasermin is a synthetic analog of IGF-1 used for the treatment of growth failure[273].

2.3.11. Nesfatin-1

2.3.11.1. overview

Nesfatin-1 is an appetite-controlling peptide secreted mainly from the hypothalamic nuclei and brain stem[274]. Nesfatin-1 was discovered in 2006 by Oh-I and colleagues who reported that nesfatin-1 suppressed food intake in mice[274]. Nesfatin is consists of 396 amino acids preceded by a 24-amino acid signal peptide[274]. Nesfatin is known to maintain approximately 85% amino acid sequence homology among rat, mouse, and human species. Nesfatin-1 is one of the few peptides currently considered for treatment of human obesity[275].

2.3.11.2. Metabolism and regulation

Besides the hypothalamus and brain stem, rat immunohistochemical results have demonstrated that the precursor of nesfatin-1, nonesterified fatty acid/nucleobinding 2 (NUCB2), is present in the pituitary gland, the forebrain and midbrain nuclei, central amygdaloid nucleus and the thoracolumbar sympathetic and sacral parasympathetic preganglionic neurons in the spinal cord[276,277]. Also, the secretion of nesfatin-1 by peripheral tissues including adipose tissue, gastric mucosa, pancreatic beta cells and testis tissue has been demonstrated[276,278]. Nesfatin-1 secretion is believed to be primarily regulated by food

patterns. For instance, 48 - hour fasting or sustained undernutrition has been reported to decrease hypothalamic nesfatin-1 mRNA and protein levels in pubertal female rats[279], whereas earlier reports indicated that mRNA and nesfatin-1 protein levels were selectively decrease in paraventricular nuclei of the hypothalamus, following 24 h fasting in adult rats[274]. Conversely, Kohno et al. have demonstrated re-feeding potently activated nesfatin-1 neurons at the PVN and SON, after a 48 - hour fast[280]. The half-life of nesfatin-1 is approximately 10 min[281]. Nesfatin-1 is known to cross the blood brain barrier in both directions after secretion, with a stability of approximately 20 minutes after intravenous injection[282].

2.3.11.3. Physiologic effects

Several lines of evidence support the notion that nesfatin-1 plays important role in the regulation of weight. For instance, injection of nesfatin-1 in the brain of leptin-receptor-mutant rats has been shown to inhibit food intake[274]. In rats, intracerebroventricular (icv) and peripheral nesfatin-1 injection has been shown to inhibit food intake[275,283]. Conversely, blockage of nesfatin-1 has been shown to increase food intake in rats[275]. Extensive studies have revealed that intravenous administration of nesfatin-1 time- and dose-dependently reduced blood glucose in hyperglycemic db/db mice[284]. Furthermore, the anti-hyperglycemic effect of nesfatin-1 in hyperglycemic db/db mice was demonstrated to be insulin-dependent[284]. Nesfatin-1 is also hypothesized to play a role in the regulation of the cardiovascular system. The intracerebrospinal injection of nesfatin-1 has been shown to elevate arterial blood pressure in rats[285]. Also, intravenous nesfatin-1 in rats has been reported to induce arterial vasoconstriction and elevate blood pressure via inhibition of NO

production[286].

2.3.12. Significance of studies

The contribution of skeletal muscle, the largest insulin-sensitive tissue in human body, in metabolic diseases, including obesity and T2DM is well-established[287]. Also, skeletal muscle is one of the few tissues directly affected by Deep Tissue Injury (DTI). Therefore, it is important to study muscle-related disorders to ensure overall health. The term MetS has been coined to summarize the cluster of risk factors that may accelerate the development of T2DM and hypertension – two leading causes of deaths worldwide. It is worth noting that skeletal muscle is linked to most risk factors of MetS including obesity, hyperglycemia, dyslipidaemia and hypertension. Since skeletal muscle accounts for nearly one-half of the body mass, skeletal muscle becomes an important target for most metabolic disorders.

Several studies have determined the fluctuations of several peptides, including ghrelin gene products and GH in MetS. Variations in circulatory GH and ghrelin gene products including UnAG, AG and obestatin have been suggested to contribute to various metabolic disorders. Ghrelin, a peptide discovered nearly 2 decades ago is the natural ligand for GHSR[139]. AG stimulates the secretion of growth hormone in response to hunger and it has been suggested to play key roles in metabolic disorders including hypertension and T2DM[288]. UnAG failed to attract attention after its discovery as it was considered an inert molecule. More so, UnAG could not bind and activate GHSR at physiological concentrations. Several studies have evaluated the possible beneficial effects of UnAG but the mechanisms mediating the

observed effects are lacking in most cases. Obestatin, the last ghrelin gene product to be identified, is currently one of the most controversial peptides. It is important to rectify the inconsistent results of obestatin actions observed by several independent groups, possibly by simultaneously quantifying UnAG, AG and ghrelin/obestatin ratios. Ultimately, investigation of the roles of ghrelin, obestatin and GH in MetS as well as UnAG in pressure ulcer may contribute to the understanding and improved management of metabolic and muscle disorders.

Although several studies suggest that GH and ghrelin gene products are related to MetS, additional research is needed to further dissect the exact roles of these peptides in relation to each diagnostic MetS risk factor, especially as the definition of MetS is confusing. Thus, it is important to investigate the roles of ghrelin gene products and GH in central-obese, hypertensive, hypertensive obese, and non-central obese normotensive individuals. Results may then be compared to subjects with different combination of MetS risk factors, to correctly interpret the roles of the peptides in different metabolic disorders.

Yoga exercise is one of the currently professed remedies for maintaining health. The results of short-term yoga studies on cardiometabolic risk factors are inconsistent in the literature. Although we have recently investigated the effects of long-term (1-year) yoga training on cardiometabolic risk factors in adults with MetS[289], there is a dearth of research focusing on the overall effects of long-term yoga on metabolic peptides, especially ghrelin gene products and GH. There is also the need to study the effects of long-term yoga exercise on estimates

of insulin resistance, for instance HOMA indices, as it is practically difficult to adopt the gold standard of insulin resistance, the euglycemic clamp method, in research studies.

A recent study has revealed that UnAG protected skeletal muscle from oxidative stress and inflammation[290] which are degenerative pathways involved in pressure ulcer. Also, a recent investigation demonstrated that a 4-day administration of UnAG in lean rats opposed the production of inflammatory cytokines and mitochondrial reactive oxygen species in gastrocnemius muscle[291]. There is need to further investigate whether UnAG could protect skeletal muscle from compression-induced damage. More importantly, there is need to identify pathways or receptors mediating the effects of UnAG, since UnAG is known to account for approximately 80% of secreted ghrelin and is currently believed to contribute to body homeostasis.

2.3.13. Study objectives

1. To clarify the synergistic or differential role of central obesity among the other MetS risk factors by examining the effects of the interaction of central obesity and the other cardiometabolic risk factors of MetS on circulatory ghrelin (AG and UnAG), obestatin, nesfatin-1, GH, and IGF-1 (Studied in Chapter 3).

2. To examine the interacting influence of hypertension and central obesity on circulatory UnAG, AG, obestatin, total ghrelin, ratios of ghrelin/obestatin, and GH (Studied in Chapter 4).

3. To investigate the effect of 1-year yoga training on metabolic peptides including UnAG, AG obestatin, GH and insulin as well as β-cell function and insulin resistance using HOMA model in the MetS older adults with central obesity (Studied in Chapter 5).

4. To test the hypothesis that UnAG protects skeletal muscle from pressure-induced injury via SIRT1 signaling pathway (Studied in Chapter 6).

Chapter 3. Obestatin and growth hormone demonstrate the interaction of central obesity and other cardiometabolic risk factors of metabolic syndrome

(Remark: The findings presented in this chapter have been submitted to *Scientific Reports* and is now under revision - Ugwu, FN, Yu AP, Tam BT, Lee PH, Ma V, Pang S, Chow AS, Cheng KK, Lai CW, Wong CS and Siu PM (2017). Obestatin and growth hormone reveal the interaction of central obesity and other cardiometabolic risk factors of metabolic syndrome)

3.1 Introduction

Central obesity, high blood pressure, hyperglycemia, and dyslipidemia are the components of metabolic syndrome (MetS)[2]. Individuals with MetS present with aberrant cardiometabolic homeostasis resulting in increased incidence of several chronic and deadly diseases such as cardiovascular diseases, diabetes mellitus and stroke. Several professional associations including National Cholesterol Education Program-Third Adult Treatment Panel III (NCEP-ATP III), World Health Organization (WHO), and International Diabetes Federation (IDF) have established diagnostic guidelines for MetS. Among the risk factors for MetS, central obesity has long been identified as an essential component of MetS[292].

Central obesity has become the focus of public attention since IDF proposed central obesity as the fundamental MetS risk factor in its revised 2005 guidelines for diagnosing MetS[34]. Compared to other MetS risk factors, central obesity is more strongly associated with different complex metabolic disorders including hypertension, insulin resistance and Type 2 diabetes Mellitus. Due to the perceived rising prevalence of central obesity, the Scottish Intercollegiate Guidelines elevated the status of central obesity from a syndrome to a disease in 2010 and described obesity as ‘a disease with multiple organ-specific consequences’[6]. Also, the American Medical Association has recognized obesity as a disease since 2013.

Results from several recent independent studies suggest that approximately 30% of the global population might become obese by the next decade. As the global financial burden

of adult obesity increases yearly, the management of childhood obesity is also on the rise, exceeding $14bn in 2010 in the United States alone[5].

Although the prevalence of central obesity is on the rise in adults across the globe, the complicated pathogenesis and mechanistic process that underlie the relationship of central obesity and MetS remains largely unknown.

Nesfatin-1, growth hormone (GH), insulin-like growth factor-1 (IGF-1) and ghrelin gene products namely ghrelin and obestatin are bioactive peptides with major influence on energy balance. Ghrelin is a peptide that circulates in the bloodstream in two principal forms namely acylated ghrelin (AG) and unacylated ghrelin (UnAG). By regulating appetite, ghrelin is known to increase mass of adipose tissue (via inducing adipogenesis) and body weight[293]. Ghrelin is linked to atherosclerosis[294] and has been shown to correlate to blood pressure[295], suggesting a role for ghrelin in cardiovascular diseases and hypertension. Obestatin, a peptid released mainly from the stomach, is encoded by the ghrelin gene and antagonizes the stimulatory effect of ghrelin on eating[137]. The fasting obestatin has been shown to be linked to insulin resistance in human and correlated negatively to body mass index and glucose level[144,296]. A recent study suggested a relationship between obestatin and blood pressure by showing that normotensive obese patients and hypertensive obese patients had a lower plasma level of obestatin when compared to the respective controls[297]. These findings suggested that obestatin might be involved in the pathogenesis of MetS which is related to obesity and high blood pressure. Nesfatin-1 is an appetite-regulating peptide that suppresses food intake[274] and has been

proposed as a potential therapeutic target for the management of obesity[275]. Besides, nesfatin-1 has been preliminarily proposed to be useful for the management of type 2 diabetes due to its dose-dependent anti-hyperglycemic effect in diabetic db/db mice[284]. The co-localization of ghrelin and nesfatin-1 in the same gastric mucosa and their differential correlation with body mass index in obese subjects have been recently reported[15]. Although the secretory regulation of orexigenic peptide, AG, and anorexigenic peptides, obestatin and nesfatin-1, from the stomach is not completely understood, GH is known to be secreted by the anterior pituitary gland with mainly anabolic but also catabolic actions in the body[298,299]. Abnormal GH level has been exhibited to increase the risk of hypertension and atherosclerotic disease[300,301]. On the other hand, enhanced accumulation of visceral adipose tissue and abnormal lipid profile have been documented in adults with GH deficiency[302,303]. IGF-1 is a peptide hormone mainly produced by the liver in response to GH and insulin stimulation and it inhibits GH secretion[304]. Circulating IGF-1 has been implicated in cardiovascular diseases as low IGF-1 level has been shown to be related to coronary artery disease which might ultimately result in fatal ischemic heart disease[305].

The definition of MetS is not unambiguous as there are various combinations of risk factors in defining MetS (e.g., MetS is diagnosed when having ≥3 risk factors according to NCEP-ATP III's definition). Between 2003 and 2004, data obtained from over 4400 U.S. adults aged 20 years or older revealed that approximately one-third of the U.S. adults were obese[306]. IDF proposed a consensus definition of MetS during the First International Congress on Prediabetes and Metabolic Syndrome in 2005, in which central obesity is considered a prerequisite for MetS. The current IDF definition of MetS is the presence of

central obesity plus any other two risk factors among elevated blood pressure, elevated triglycerides, elevated fasting blood glucose and reduced high-density lipoprotein-cholesterol (HDL-C)[34]. The IDF diagnostic criteria for MetS was adopted in the evaluation of data of over 15,000 subjects pooled from nine studies participating in the Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe (DECODE) study[307]. Although the MetS prevalence and independent MetS risk factors have been described by several studies, interpretations that support central obesity as an essential key risk factor of MetS, as proposed by IDF, are limited. This is related to the perplexing definitions of MetS by relevant authorities that diagnose MetS via a combination of 3, 4 or 5 independent risk factors. For instance, the DECODE study stated that "Subjects with the IDF MetS were, by definition, more obese and also had higher blood pressure than the non-obese individuals with 3 – 4 abnormalities; but the lipid profiles were more atherogenic in the later than in the former"[307]. The above statements are somehow difficult to interpret because the other two or more MetS risk factors besides central obesity were not specified and it is difficult to categorize individuals with only 3 MetS risk factors and individuals with 4 MetS risk factors in the same MetS-diagnosed group (due to the different combination possibilities).

Despite the consequences of central obesity coupled with its rising prevalence, the role of central obesity is often obscured by the presence of other risk factors. More so, there is a dearth of research on the interaction of central obesity and the other MetS risk factors.

Given the current epidemic of cardio- and cerebro-vascular diseases which are related to obesity and MetS, understanding the role of obesity in MetS would be important. Therefore, this study sought to clarify the synergistic or differential role of central obesity among the other MetS risk factors by examining the effects of the interaction of central obesity and the other cardiometabolic risk factors of MetS on circulatory ghrelin (AG and UnAG), obestatin, nesfatin-1, GH, and IGF-1.

3.2 Methods

3.2.1. Subjects and MetS parameters

Based on specific criteria for MetS parameters defined by NCEP-ATP III, serum and MetS data of 133 Hong Kong Chinese adults of both sexes with age ranged 24 to 86 years were retrieved from a total of 1492 archived data of participants screened between November 2010 and August 20131. In this study, the included subjects had no MetS risk factor (n = 53), no MetS risk factor except central obesity (n = 33), all 5 MetS risk factors except central obesity (n = 10) or all 5 MetS risk factors including central obesity (n = 37) as defined by NCEP-ATP III. According to NCEP-ATP III[51], MetS is diagnosed in individuals with more than two of the characteristics: (1) central obesity as indicated by waist circumference exceeding 90 cm or 80 cm for Asian males or females, respectively, (2) high blood pressure defined as systolic blood pressure (SBP) above 130 mmHg or diastolic blood pressure (DBP) above 85 mmHg, (3) fasting blood glucose (FBG) above 100 mg/dL, (4) elevated plasma triglycerides (TG) exceeding 150 mg/dL, and (5) low level of high-density lipoprotein cholesterol (HDL-C) less than 40 mg/dL or 50 mg/dL for males

or females, respectively. All subjects were screened and individuals with dementia or mental disorders, severe or acute cardiovascular diseases, post-stroke, neuromusculoskeletal illness, acute medical illness, symptomatic heart or lung diseases, severe rheumatoid arthritis, osteoarthritis or pulmonary illness and participants who were immobile, smoker or under treatment for metabolic abnormalities were excluded. To investigate the contribution of central obesity in MetS, a 2 x 2 factorial research design was adopted to evaluate the interaction of central obesity with the cluster of the other four MetS parameters namely elevated blood pressure, elevated triglycerides, elevated fasting blood glucose and reduced HDL-C. Data were analyzed by classifying the participants into: (A) absence of the cluster of four MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C) vs. presence of the cluster of four MetS risk factors; (B) non-central obese vs. central obese; (C) NRFNO – no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity, NRFO – no MetS risk factors but with central obesity, RFNO – with the cluster of the four MetS risk factors but without central obesity or RFO – with the cluster of the four MetS risk factors plus central obesity. Blood pressure (systolic and diastolic) was determined by an electronic blood pressure monitor (Accutorr Plus, Datascope). Waist circumference was measured with an inelastic measuring tape by trained personnel. Fasting venous blood samples were obtained after at least 10 hours fast by certified phlebotomists for the measurements of glucose, triglycerides and high-density lipoprotein cholesterol (HDLC) using an automatic clinical chemistry analyser (Architect CI8200, Abbott Diagnostics). All samples were aliquoted and stored at

-80°C until needed for analysis. Human research ethics approval was obtained from the human subject ethics subcommittee of the Hong Kong Polytechnic University (ethics approval number: HSEARS20150203002) and written informed consent was obtained from the subjects prior to commencement of the experiment. All methods were performed in accordance with the relevant guidelines and regulations.

3.2.2. Peptide measurement

Sera from the obtained venous blood samples were used to measure UnAG, AG, obestatin, nesfatin-1, GH and IGF-1 by commercially available Enzyme-linked immunosorbent assay (ELIZA) kits. All protocols were in accordance with manufacturers´ recommendations.

UnAG and AG: UnAG and AG ELISA kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RA194063400R and RA194062400R, respectively). The assays were based on a double-antibody sandwich technique. The wells of each human UnAG or AG antibody-coated microplate were rinsed before dispensing standards, dilution buffer, samples, quality controls and conjugate solution (blank) into appropriate wells. Plates were covered and incubated for 20 hours at 4°C. Plate contents were discarded, rinsed adequately and blotted on paper towels to remove liquid traces. Substrate solution (Ellman's Reagent) was added to each well and the plate was read at 410 nm using a spectrophotometer. The plate was checked periodically every 30 minutes until maximum absorbance was attained. The results were calculated after the average of the blank readings were subtracted from each well.

Obestatin: obestatin kits were purchased from Biomatik Corporation, Canada (catalog no: EKU06381). The microplates provided in the kits were pre-coated with an antibody specific to obestatin. Firstly, 100µL of standards and samples were added to appropriate microplate wells with a biotin-conjugated antibody specific to obestatin. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated for 1 hour at 37°C. Wells were aspirated and 100µL of Detection Reagent A was introduced into each well. Plates were incubated again for 1 hour at 37°C. Wells were aspirated, washed 3 times and 100µL of Detection Reagent B was added to each well, followed by incubation of plates for 30 minutes, at 37°C. Wells were aspirated and washed 5 times; 90µL of Substrate Solution was added to the wells and plates were incubated for 20 minutes, at 37°C. After TMB Substrate Solution was added, only wells that contained obestatin, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. After incubation, 50µL of sulphuric acid Stop Solution was added to each well and plates were immediately read at 450nm using a spectrophotometer. A standard curve was plotted and the concentration of obestatin in the samples was then determined by comparing the optical density (OD) of the samples to the standard curve.

Nesfatin-1: nesfatin-1 kits were purchased from Biomatik Corporation, Canada (Catalog no: EKU06180). The assay was based on the competitive inhibition enzyme immunoassay technique.

Microplates were pre-coated with a monoclonal antibody specific to nesfatin-1. Firstly, 50µL of standards, samples and blank were added to appropriate wells. Next, 50µL of

Detection Reagent A was added to each well immediately using a multichannel pipette. The contents of each well were gently mixed via a microplate shaker. Plates were covered with a plate sealer and incubated for 1 hour at 37°C. Plates were aspirated, washed 3 times and blotted against absorbent paper. This was followed by addition of 100µL of Detection Reagent B to each well. Plates were again sealed with a plate sealer and incubated for 30 minutes at 37°C. Plates were aspirated, washed 5 times and blotted against absorbent paper. Thereafter, 90µL of Substrate Solution was added to each well. Plates were covered with a new plate sealer and incubated for 20 minutes in the dark, at 37°C. Following the addition of Substrate Solution, the colour of the liquid in each well turned blue. Finally, 50µL of Stop Solution was added to each well. The colour of the liquid in each well changed to yellow after addition of Stop Solution. Each plate was read with a microplate reader at 450nm. A standard curve was plotted and the concentration of nesfatin-1 in the samples was then determined by comparing the OD of the samples to the standard curve.

GH: GH kits were purchased from Abcam, Cambri (Catalog no: ab190811). The SimpleStep ELISA® included an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocaptured the sample analyte in solution. The entire complex (capture antibody/analyte/detector antibody) was immobilized via immunoaffinity of an anti-tag antibody coating the wells of each plate. The assay began with addition of 50 µL of standard and samples to appropriate wells. This was followed by the addition of 50 µL of the Antibody Cocktail to each well. Next, plates were sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. After incubation,

wells were washed 3 times to remove unbound materials. After the last wash, plates were inverted and blotted against clean paper. Thereafter, 100 µL of TMB Substrate was added to each well and incubated for 10 minutes in the dark on a plate shaker set to 400 rpm. The reaction was terminated by addition of Stop Solution which completed the color change from blue to yellow. Colour signals generated was proportional to the amount of bound analyte and the intensity was measured spectrophotometrically at 450 nm. A standard curve was plotted and the concentration of GH in the samples was determined by comparing the OD of the samples to the standard curve.

IGF-1: IGF-1 kits were purchased from Abcam, Cambri (Catalog no: ab100545). This assay employed an antibody specific for Human IGF-1 pre-coated on a 96-well plate. Firstly, 100 µL of standards and samples were pipetted into the wells and IGF-1 present in a solution was bound to the wells by the immobilized antibody. Plates were sealed and incubated over night at 4°C with gentle shaking. Next, well contents were discarded and wells were washed 4 times. After the last wash, plates were inverted and blotted against clean paper towels to remove any remaining liquid. Thereafter, 100 µL of Biotinylated IGF-1 Detection Antibody was added to each well, followed by incubation of plates for 1 hour at room temperature with gentle shaking. The solutions were discarded. Wells were again washed 4 times and after the last wash, plates were inverted and blotted against clean paper towels to remove any remaining liquid. After washing away unbound biotinylated antibody, 100 µL of HRP-conjugated streptavidin was pipetted to the wells. Plates were incubated for 45 minutes at room temperature with gentle shaking. Wells were washed 4

times and blotted against clean paper towels. Thereafter, 100 μL of TMB One-Step Substrate Reagent was added to each well and this was quickly followed by incubation of plates for 30 minutes at room temperature, in the dark with gentle shaking. Finally, 50 μL of Stop Solution was added to each well and plates were read at 450 nm via a spectrophotometer. The addition of Stop Solution changed the colour of liquid in each well from blue to yellow, and the intensity of the color was proportional to the amount of IGF-1 present in the sample or standard. A standard curve was plotted for each plate and the concentration of GH in the samples was determined by comparing the OD of the samples to the standard curve.

3.2.3. Statistical analysis

Data are expressed as mean ± standard deviation. The main effect of central obesity, the main effect of the cluster of the other four MetS risk factors and the interaction of central obesity with the cluster of the other four MetS risk factors on UnAG, AG, total ghrelin, obestatin, nesfatin-1, GH and IGF-1 and the ratio of obestatin/ghrelin were analyzed by generalized estimating equations (GEE)[308]. GEE was adopted as it has less stringent requirement on normality assumption. The differences between two groups were detected by Mann-Whitney U Test whereas statistical differences among the four groups were determined by Kruskal-Wallis H Test, followed by Dunn-Bonferroni post hoc tests[309]. Spearman's correlation analysis was performed to examine the correlations between the peptide concentrations and the MetS parameters. Statistical significance was accepted at $p < 0.05$. All statistical procedures were conducted using the Statistical Package for the Social

Sciences (SPSS) version 22 for Windows.

3.3. Results

3.3.1. Obestatin but not ghrelin revealed the interaction of central obesity with the cluster of the other four MetS risk factors

The level of UnAG was 32% (mean difference = 108 pg/mL) significantly reduced in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors (Fig. 3.1A). UnAG tended to be 12% lower in central obese subjects when compared to non-central obese subjects (Fig. 3.1B). Central obesity and the cluster of the other four MetS risk factors had no interaction effect on UnAG (Fig. 3.1C). The main effect of central obesity on UnAG was not significant. However, the main effect of the cluster of the other four MetS risk factors on UnAG ($p = 0.001$) was statistically significant (Fig. 3.1C). Kruskal-Wallis H Test showed a significant difference in UnAG among NRFNO, NRFO, RFNO and RFO groups (Fig. 3.1C). Post hoc analyses showed significant differences in UnAG between NRFNO and RFNO, NRFNO and RFO and between NRFO and RFO groups (Fig. 3.1C).

AG was 49% (mean difference = 3.2 pg/mL) significantly lower in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors (Fig. 3.1D). AG tended to be 12% lower in central obese subjects compared to non-central obese subjects (Fig. 3.1E). Central obesity and the cluster of the other four MetS risk factors had no interaction effect on AG (Fig. 3.1F). The main effect of central obesity on

AG was not significant. However, the main effect of the cluster of the other four MetS risk factors on AG ($p = 0.001$) was statistically significant (Fig. 3.1F). Kruskal-Wallis H Test showed a significant difference in AG among NRFNO, NRFO, RFNO and RFO groups (Fig. 3.1F). Post hoc analyses showed significant differences in AG between NRFNO and RFNO, NRFNO and RFO and between NRFO and RFO groups (Fig. 3.1F).

Total ghrelin was 32% (mean difference = 111.3 pg/mL) significantly reduced in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors (Fig. 3.1G). When compared to non-central obese subjects, total ghrelin tended to be 12% lower in central obese subjects (Fig. 3.1H). Central obesity and the cluster of the other four MetS risk factors had no interaction effect on total ghrelin (Fig. 3.1I). The main effect of central obesity on total ghrelin was not significant. However, the main effect of the cluster of the other four MetS risk factors on total ghrelin ($p = 0.001$) was statistically significant (Fig. 3.1I). Kruskal-Wallis H Test showed a significant difference in total ghrelin among NRFNO, NRFO, RFNO and RFO groups (Fig. 3.1I). Post hoc analyses showed significant differences in total ghrelin between NRFNO and RFNO, NRFNO and RFO and between NRFO and RFO groups (Fig. 3.1I).

In contrast to UnAG, AG and total ghrelin, obestatin was 116% (mean difference = 22 ng/mL) significantly higher in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors. (Fig. 3.1J). Also, obestatin was 123% (mean difference = 20 ng/mL) significantly higher in central obese subjects when compared with non-central obese subjects (Fig. 3.1K). Central obesity and the cluster of

the other four MetS risk factors had interaction effect ($p = 0.001$) on obestatin (Fig. 3.1L). Comparing with the subjects with no risk factors, those with only the cluster of four MetS risk factors had no difference in obestatin ($p = 0.901$), those with only central obesity had higher obestatin (mean difference = 7.4 ng/mL, $p = 0.009$) whereas the subjects with all risk factors had higher obestatin (mean difference = 31.3 ng/mL, $p = 0.001$). The main effect of central obesity ($p = 0.001$) and the main effect of the cluster of the other four MetS risk factors ($p = 0.001$) on obestatin were also statistically significant (Fig. 3.1L). In addition, Kruskal-Wallis H Test showed a significant difference in obestatin among the four groups under study. Further analyses showed significant differences between NRFNO and RFNO, NRFNO and RFO, NRFO and RFO and between RFNO and RFO groups (Fig. 3.1L).

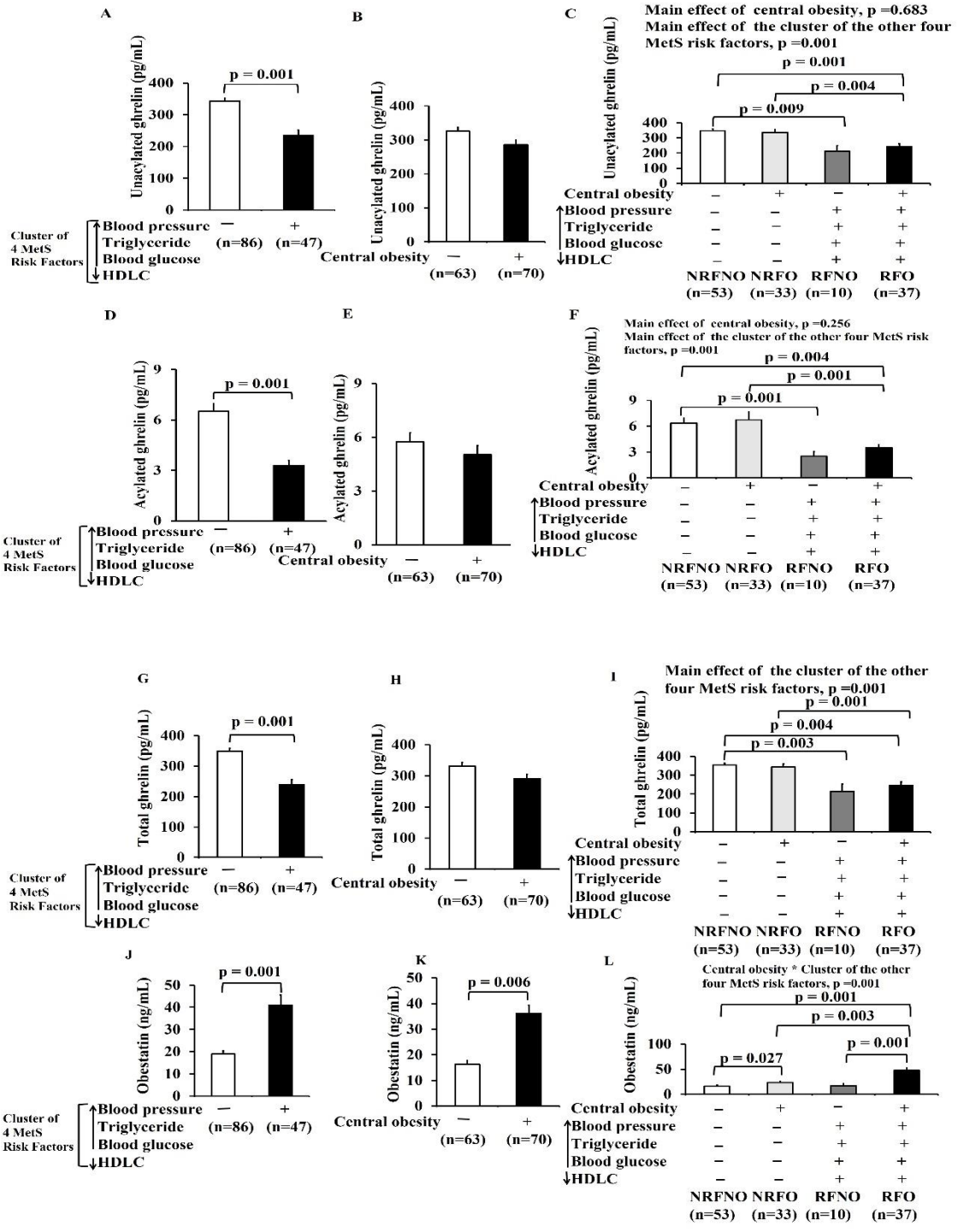

Figure 3.1 Ghrelin gene products in normal subjects and individuals with central obesity with or without the cluster of the other four MetS risk factors

Unacylated ghrelin, acylated ghrelin and total ghrelin were decreased in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (A, D, G). Unacylated ghrelin, acylated ghrelin and total ghrelin tended to be decreased in central obese individuals (B, E, H). Subjects with all MetS risk factor with/without central obesity had lower unacylated ghrelin, acylated ghrelin and total ghrelin levels (C, F, I). Obestatin increased in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (J). Obestatin increased in subjects with central obesity (K). Obestatin revealed the interaction of central obesity with the cluster of the other four MetS risk factors and subjects with central obesity with/without the cluster of the other four MetS risk factors had higher obestatin levels (L). The main effect of central obesity, the main effect of the cluster of the other four MetS risk factors and the interaction of central obesity with the cluster of the other four MetS risk factors were analyzed with generalized estimating equations. Significance level was set at $p < .05$

NRFNO – no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity; NRFO – no MetS risk factors but with central obesity; RFNO – with the cluster of the four MetS risk factors but without central obesity; RFO – with the cluster of the four MetS risk factors plus central obesity.

3.3.2. Ratios of ghrelin gene products reflect the interaction of central obesity with the cluster of the other four MetS risk factors

Changes in the ratios of ghrelin gene products have been suggested as important indices for diet-related disorders including anorexia nervosa, bulimia nervosa and inflammatory bowel diseases[143,174,310]. In the current study, the ratio of obestatin/UnAG was significantly higher in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors by 4-fold (Fig. 3.2A. Similarly, the ratio of obestatin/UnAG was significantly higher in central obese subjects when compared to non-central obese subjects by 3.2-fold (Fig. 3.2B). Central obesity and the cluster of the other four MetS risk factors had interaction effect ($p = 0.028$) on the ratio of obestatin/UnAG (Fig. 3.2C). Comparing with the subjects with no risk factors, those with only the cluster of four MetS risk factors had no difference in obestatin/UnAG ratio ($p = 0.143$) while those with only central obesity had higher ratio of obestatin/UnAG ratio (mean difference = 33, $p = 0.005$) whereas the subjects with all risk factors had higher obestatin/UnAG ratio (mean difference = 234.7, $p = 0.001$). The main effect of central obesity on the ratio of obestatin/UnAG ($p = 0.001$) was statistically significant. Also, the main effect of the cluster of the other four MetS risk factors on the ratio of obestatin/UnAG ($p = 0.001$) was statistically significant (Fig. 3.2C). Kruskal-Wallis H Test showed there were significant differences in the ratio of obestatin/UnAG among the four groups (Fig. 3.2C). Post-hoc tests revealed significant differences in the ratio of obestatin/UnAG between NRFNO and RFO and between NRFO and RFO groups (Fig. 3.2C).

The ratio of obestatin/AG was significantly higher in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors by 3.8-fold (Fig. 3.2D). Similarly, the ratio of obestatin/AG was significantly higher in central obese subjects when compared to non-central obese subjects by 2.5-fold (Fig. 3.2E). Central obesity and the cluster of the other four MetS risk factors had interaction effect ($p = 0.003$) on the ratio of obestatin/AG (Fig. 3.2F). Comparing with the subjects with no risk factors, those with only the cluster of four MetS risk factors had no difference in obestatin/AG ratio ($p = 0.447$) while those with only central obesity had higher ratio of obestatin/AG ratio (mean difference = 4760.8, $p = 0.029$) whereas the subjects with all risk factors had higher obestatin/AG ratio (mean difference = 14838.4, $p = 0.001$). The main effect of central obesity on the ratio of obestatin/AG ($p = 0.001$) was statistically significant. Also, the main effect of the cluster of the other four MetS risk factors on the ratio of obestatin/AG ($p = 0.001$) was statistically significant (Fig. 3.2F. Kruskal-Wallis H Test showed there were significant differences in the ratio of obestatin/AG among the four groups (Fig. 3.2F). Post-hoc tests revealed significant differences in the ratio of obestatin/AG between NRFNO and RFO and between NRFO and RFO groups (Fig. 3.2F).

The ratio of obestatin/total ghrelin was significantly higher in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors by 4-fold (Fig. 3.2G). Similarly, the ratio of obestatin/total ghrelin was significantly higher in central obese subjects when compared to non- central obese subjects by 3.2-fold (Fig. 3.2H). Central obesity and the cluster of the other four MetS risk factors had interaction

effect (p = 0.026) on the ratio of obestatin/total ghrelin (Fig. 3.2I). Comparing with the subjects with no risk factors, those with only the cluster of four MetS risk factors had no difference in obestatin/total ghrelin ratio (p = 0.139) while those with only central obesity had higher ratio of obestatin/total ghrelin ratio (mean difference = 32, p = 0.005) whereas the subjects with all risk factors had higher obestatin/total ghrelin ratio (mean difference = 230, p = 0.001). The main effect of central obesity on the ratio of obestatin/total ghrelin (p = 0.001) was statistically significant. Also, the main effect of the cluster of the other four MetS risk factors on the ratio of obestatin/total ghrelin (p = 0.001) was statistically significant (Fig. 3.2I). Kruskal-Wallis H Test showed there were significant differences in the ratio of obestatin/total ghrelin among the four groups (Fig. 3.2I). Post-hoc tests revealed significant differences in the ratio of obestatin/total ghrelin between NRFNO and RFO and between NRFO and RFO groups (Fig. 3.2I).

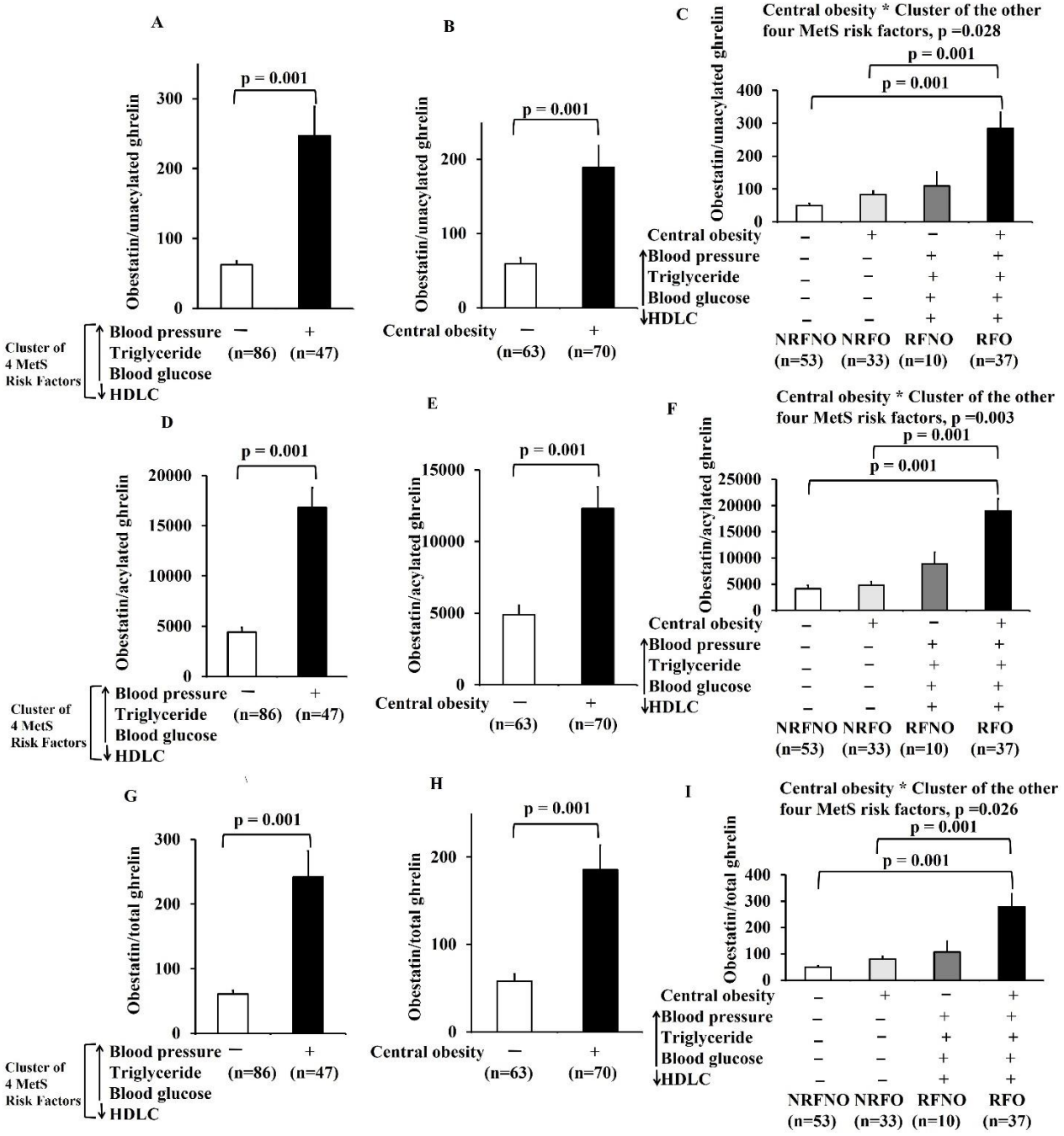

Figure 3. 2 Ratios of ghrelin gene products in normal subjects and individuals with central obesity with or without the cluster of the other four MetS risk factors

The ratios of obestatin/unacylated ghrelin, obestatin/acylated ghrelin and obestatin/total ghrelin increased in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (A, D, G). The ratios of obestatin/unacylated ghrelin, obestatin/acylated ghrelin and obestatin/total ghrelin increased in subjects with central obesity (B, E, H). The interaction of central obesity with the cluster of the other four MetS risk factors was reflected by the ratios of ghrelin gene products (C, F, I). Subjects with all MetS risk factors with/without central obesity had higher obestatin/unacylated ghrelin, obestatin/acylated ghrelin and obestatin/total ghrelin ratios (C, F, I). The main effect of central obesity, the main effect of the cluster of the other four MetS risk factors and the interaction of central obesity with the cluster of the other four MetS risk factors were analyzed with generalized estimating equations. Significance level was set at $p < .05$

NRFNO – no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity; NRFO – no MetS risk factors but with central obesity; RFNO – with the cluster of the four MetS risk factors but without central obesity; RFO – with the cluster of the four MetS risk factors plus central obesity.

3.3.3 GH but not Nesfatin-1 or IGF-1 revealed the interaction of central obesity with the cluster of the other four MetS risk factors

Nesfatin-1 tended to be higher in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors, p=0.06 (Fig. 3.3A). Nesfatin-1 tended to be 14% higher in central obese subjects in comparison to non-central obese subjects (Fig. 3.3B). The interaction of central obesity with the cluster of the other four MetS risk factors was not exhibited by nesfatin-1 according to our two-way ANOVA results (Fig. 3.3C). There was no main effect of central obesity or the cluster of the other four MetS risk factors on nesfatin-1 (Fig. 3.3C). Kruskal-Wallis H Test showed no significant difference in nesfatin-1 among NRFNO, NRFO, RFNO and RFO groups (Fig. 3.3C).

GH was 79% (mean difference = 756.9 pg/mL) significantly lower in subjects with the cluster of four MetS risk factors in comparison to subjects without the cluster of four MetS risk factors. (Fig. 3.3D). GH was 71% (mean difference = 792.3 pg/mL) significantly lower in central obese individuals compared to non-central obese subjects (Fig. 3.3E). Central obesity and the cluster of the other four MetS risk factors had interaction effect (p = 0.001) on GH (Fig. 3.3F). Comparing with the subjects with no risk factors, those with only central obesity had lower GH (mean difference = 846.3, p = 0.001). GH was further decreased in subjects with the cluster of four MetS risk factors and all risk factors (mean differences = 1103.9 and 1075.7 and p = 0.001 and 0.001, respectively) when compared to the subjects with no risk factors. The main effect of central obesity (p = 0.001) and the main effect of

the cluster of the other four MetS risk factors ($p = 0.001$) on GH were also statistically significant (Fig. 3.3F). There was significant difference in GH among the four groups under study (Fig. 3.3F). Post-hoc tests showed significant difference in GH between NRFNO and NRFO, NRFNO and RFNO, NRFNO and RFO and between NRFO and RFO groups (Fig. 3F). There was neither significant difference in IGF-1 level between subjects with the cluster of four MetS risk factors and subjects without the cluster of four MetS risk factors. (Fig. 3.3G) nor between central obese and non-central obese subjects (Fig. 3.3H). There was no interaction of central obesity with the cluster of the other four MetS risk factors on IGF-1 (Fig. 3.3I). Also, there was no main effect of central obesity or the cluster of the other four MetS risk factors on IGF-1 (Fig. 3.3I). Furthermore, there was no significant difference in IGF-1 between NRFNO, NRFO, RFNO and RFO groups (Fig. 3.3I).

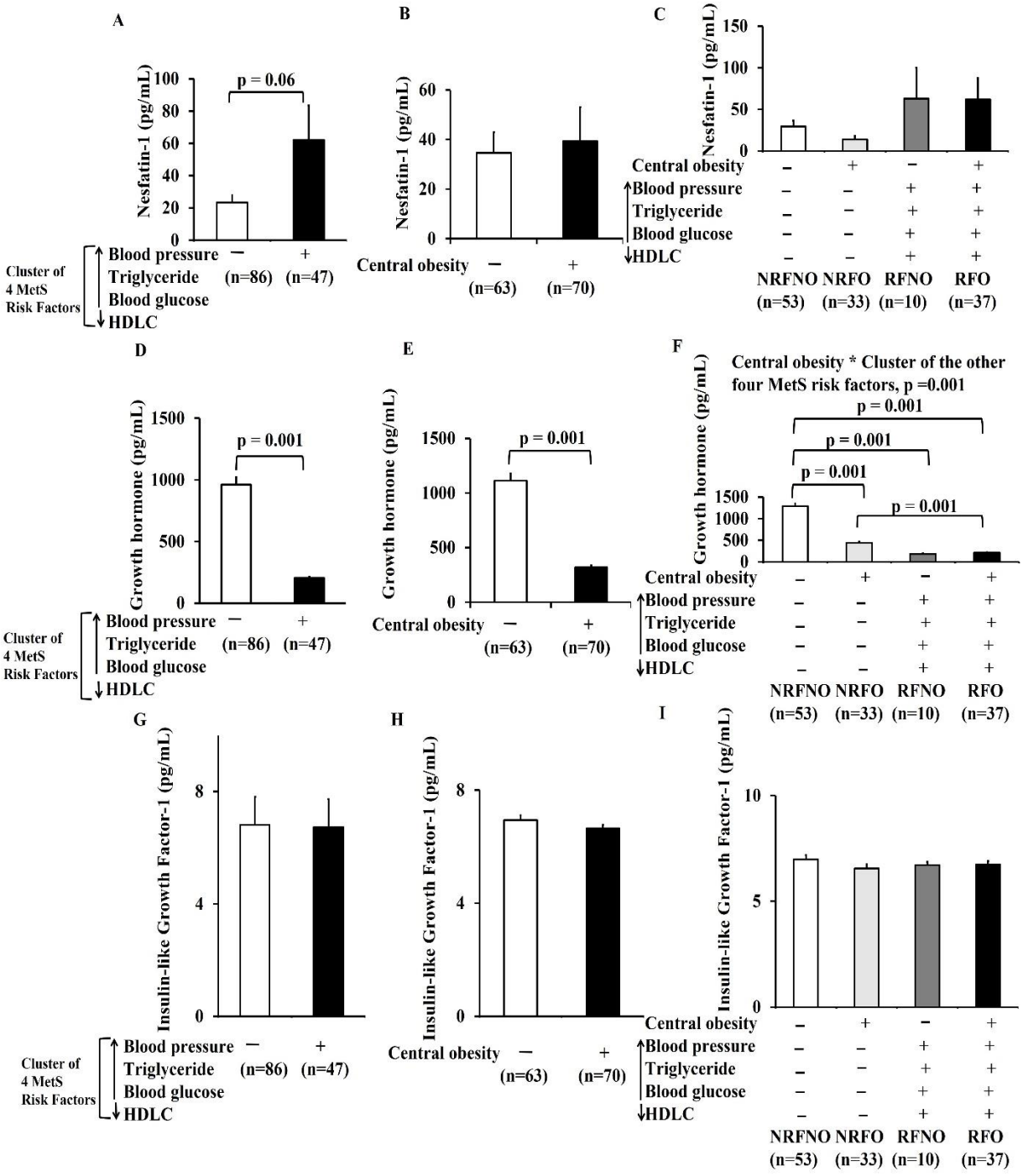

Figure 3. 3 Nesfatin-1, growth hormone and IGF-1 in normal subjects and individuals with central obesity with or without the cluster of the other four MetS risk factors

Nesfatin-1 tended to increase in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (A). Nesfatin-1 tended to increase in subjects with central obesity(B). Nesfatin-1 did not reveal the interaction of central obesity with the cluster of the other MetS risk factors. Subjects with all MetS risk factors with/without central obesity tended to exhibit higher nesfatin-1 levels (C). Growth hormone decreased in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (D). Growth hormone decreased in subjects with central obesity (E). The interaction of central obesity with the cluster of the other four MetS risk factors was revealed by growth hormone (F). Subjects with all MetS risk factors with/without central obesity had lower growth hormone levels. Insulin-like growth factor-1 was not altered in subjects with the cluster of four MetS risk factors regardless of the status of central obesity (G). Insulin-like growth factor-1 was not altered in individuals with central obesity (H). Insulin-like growth factor-1 was not altered in irrespective of the cardiometabolic risk factor (I). The main effect of central obesity, the main effect of the cluster of the other four MetS risk factors and the interaction of central obesity with the cluster of the other four MetS risk factors were analyzed with generalized estimating equations. Significance level was set at $p < .05$

NRFNO – no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity; NRFO – no MetS risk factors but with central obesity; RFNO – with the cluster of the four MetS risk factors but without central obesity; RFO – with the cluster of the four MetS risk factors plus central obesity.

3.3.4 Relationship of biomarker with cardiometabolic risk factors

The associations of UnAG, AG, total ghrelin, obestatin, nesfatin-1, GH and IGF-1 with the individual cardiometabolic risk factors are shown in Figure 3.4. Spearman's correlation showed statistically significant negative correlations between UnAG with systolic blood pressure (r = -0.42, p = 0.001), UnAG with diastolic blood pressure (r = -0.21, p = 0.017), UnAG with waist circumference (r = -0.33, 0.001), UnAGwith triglycerides (r = -0.41, p = 0.001), UnAG with fasting blood glucose (r = -0.39, p =0.001), AG with systolic blood pressure (r = -0.48, p = 0.001), AG with diastolic blood pressure (r = -0.32, p = 0.001), AG with waist circumference (r = -0.18, p = 0.038), AG with triglycerides (r = -0.32, p = 0.001), AG with fasting blood glucose (r = -0.41, p = 0.001), total ghrelin with systolic blood pressure (r = -0.43, p = 0.001), total ghrelin with diastolic blood pressure (r = -0.21, p = 0.014), total ghrelin with waist circumference (r = -0.34, p = 0.001), total ghrelin with triglycerides (r = -0.41, p = 0.001) and total ghrelin with fasting blood glucose(r = -0.40, p = 0.001) but statistically significant positive correlations between UnAG with HDL-C (r = 0.42, p= 0.001), AG with HDL-C (r = 0.31, p = 0.001) and total ghrelin with HDL-C (r = 0.42, p = 0.001). Conversely, obestatin revealed statistically significant positive correlation with systolic blood pressure (r = 0.36, p = 0.001), diastolic blood pressure (r = 0.21, p = 0.017), waist circumference (r = 0.44, p = 0.001), triglycerides (r = 0.33, p = 0.001) and fasting blood glucose (r = 0.32, p = 0.001) but statistically significant negative correlation with HDL-C (r = -0.31, p = 0.001).

GH showed statistically significant negative correlations with systolic blood pressure (r = -0.67, p = 0.001), diastolic blood pressure (r = -0.58, p = 0.001), waist circumference (r =

-0.65, p = 0.001), triglycerides (r = -0.66, p = 0.001) and fasting blood glucose (r = -0.64, p = 0.001) but statistically significant positive correlation with HDL-C (r = 0.65, p = 0.001). Significant positive association was observed between nesfatin-1 and triglycerides (r = 0.28, p=0.001). There was no correlation between IGF-1 and all cardiometabolic risk factors (Fig. 3.4).

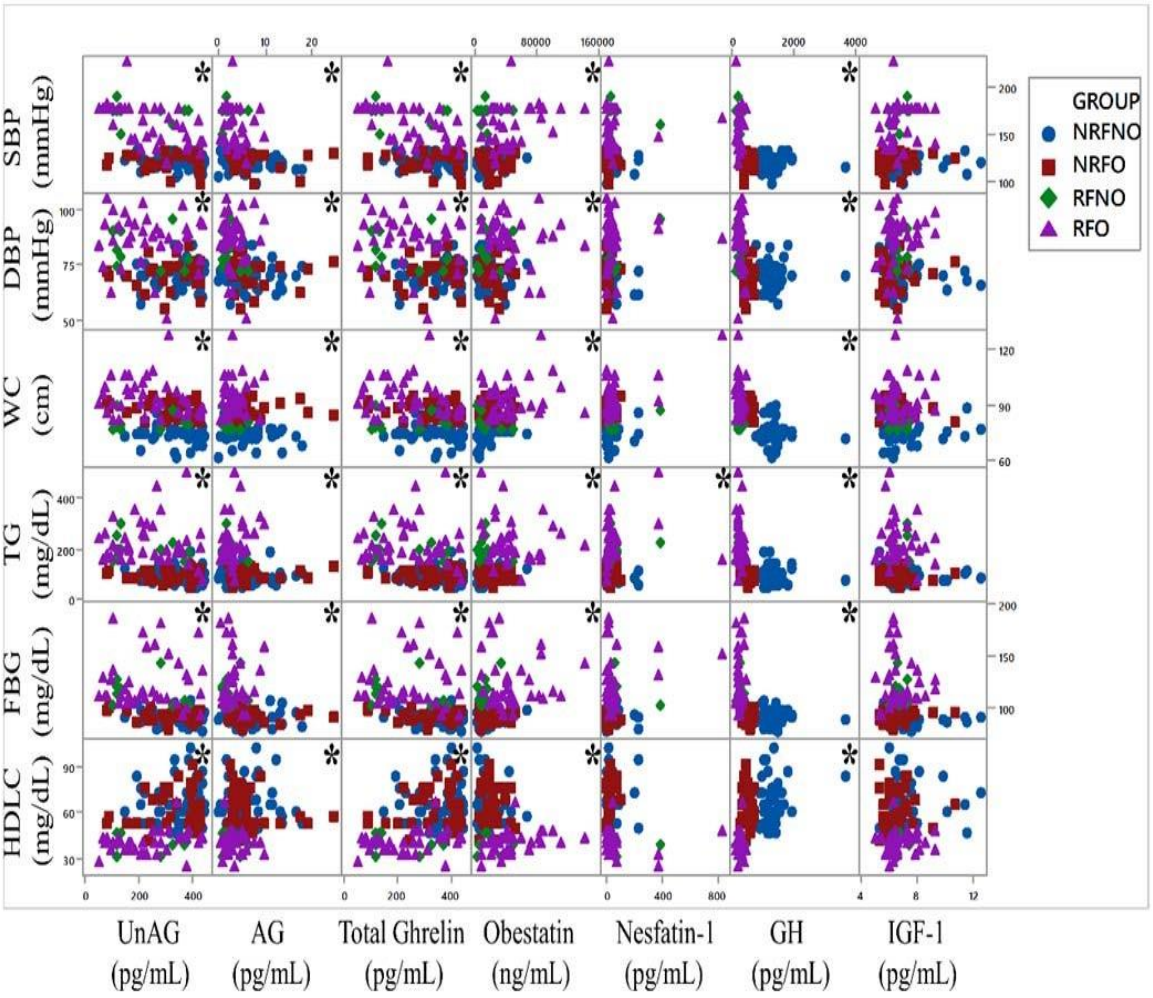

Figure 3. 4 Matrix plot of ghrelin gene products, nesfatin-1, GH, IGF-1 and cardiometabolic risk factors

UnAG, AG, total ghrelin and GH were positively correlated with HDLC only but negatively correlated with the remaining cardiometabolic risk factors (SBP, DBP, WC, TG and FBG). Obestatin correlated negatively with HDLC but correlated positively with all other MetS risk factors. Nesfatin-1 correlated positively with TG alone whereas, IGF-1 showed no correlation with all MetS risk factors. The plots suggest that a linear association exists between the number of cardiometabolic risk factors and ghrelin gene products and growth hormone i.e Subjects with the cluster of four (RFNO group) or cluster of all five (RFO group) cardiometabolic risk factors had lower levels of UnAG, AG, total ghrelin and GH compared with subjects who had only one (NRFO group) or no (NRFNO) cardiometabolic risk factor. Conversely, individuals with the cluster of four or cluster of all five cardiometabolic risk factors had higher levels of obestatin compared with individuals who had only one or no cardiometaboilc risk factor. UnAG, unacylated ghrelin; AG,acylated ghrelin; SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; TG, triglycerides; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; NRFNO, no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity) group; NRFO, no MetS risk factors but with central obesity group; RFNO, with the cluster of the four MetS risk factors but without central obesity group; RFO, with the cluster of the four MetS risk factors plus central obesity. Spearman's correlation was employed for the association studies. * $p < 0.05$.

NRFNO – no MetS risk factors (including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose, reduced HDL-C and central obesity; NRFO – no MetS risk factors but with central obesity; RFNO – with the cluster of the four MetS risk factors but without central obesity; RFO – with the cluster of the four MetS risk factors plus central obesity.

3.4. Discussion

Understanding the constellation of factors associated with MetS might provide insights on the associated mechanistic pathways and assist health care professionals in exploring regimens for the prevention of MetS. The NCEP-ATP III prioritized central obesity in the diagnosis of MetS[292] and in 2005, IDF revised its diagnostic criteria by emphasizing on central obesity as determined by waist circumference as the sine qua non diagnostic criterion of MetS[34]. The present study attempted to reveal the interaction of central obesity and the cluster of the other four MetS risk factors (namely raised blood pressure, raised triglycerides, raised fasting blood glucose and reduced HDL cholesterol) on circulatory peptides including UnAG, AG, obestatin, nesfatin-1, GH and IGF-1. Our results illustrated the interaction of central obesity and the other four MetS factors as manifested by obestatin, obestatin/ghrelin ratios and GH. Obestatin was increased in central obese subjects compared to non-central obese subjects regardless of the status of the other four MetS risk factors. When compared with subjects with no MetS risk factor or only central obesity, GH was decreased in subjects with the cluster of the other four MetS risk factors irrespective of the presence or absence of central obesity. Furthermore, UnAG, AG, total ghrelin and GH were reduced in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors regardless of the status of central obesity. Conversely, nesfatin-1 was elevated in subjects with the cluster of four MetS risk factors compared to subjects without the cluster of four MetS risk factors regardless of the status of central obesity. Significant associations between ghrelin gene products and GH and the

independent MetS risk factors were also found.

Ghrelin, a peripheral hunger hormone and a main regulator of energy balance is receiving a great deal of focus in energy-related disorders. Our findings that the fasting circulatory levels of UnAG, AG and total ghrelin were reduced in subjects with central obesity or the cluster of the other four MetS risk factors when compared to corresponding control subjects are in accordance with an earlier study reporting that the total plasma ghrelin concentration was significantly decreased in obese subjects with insulin resistance relative to the normal healthy subjects[311]. It is important to note that the conclusion from this investigation was based on the measurement on the total ghrelin which included both acylated and unacylated forms of ghrelin. Here, we further clarified the independent associations of UnAG and AG ghrelin individually with the risk factors of MetS. The separate examination of the two forms of ghrelin is indeed necessary for gaining a complete understanding because UnAG and AG might act in the same or opposite fashion[188,312]. Generally, ghrelin is believed to increase body weight and adiposity by inducing adipogenesis[293]. Our study identified a connection between central obesity and the regulation of ghrelin, in which ghrelin was not found to be invariably suppressed in central obese individuals. However, UnAG, AG and total ghrelin was observed to be consistently suppressed among those subjects with the cluster of four MetS risk factors regardless of central obesity. Our data showed that AG was not significantly different between non-obese and obese subjects, which is contrary to the findings of Tschop et al. reporting a significant decrease in fasting plasma AG in overall obese individuals in comparison to lean individuals[145]. The authors studied overall obesity in the subjects using body mass index (BMI as the diagnostic tool. It is noteworthy

that several studies proposed the measurement of waist circumference rather than BMI in the diagnosis of MetS and related disorders including type 2 diabetes and cardiovascular diseases[313,314]. Thus, it is plausible that the observed difference between our study and research by Tschop et al. might be related to the nature of obesity studied (central versus overall) as well as the parameter employed in the definition of obesity (waist circumference versus BMI). Our correlation analyses corroborated previous reports that ghrelin was negatively correlated to blood pressure[294,295]. It is probable that the higher blood pressure in relation to lower ghrelin level in obese individuals or subjects with the cluster of the other four MetS risk factors compared to their respective controls was associated with a larger waist circumference which has been identified as a strong risk factor for hypertension[315]. Also, the intimate relationship between central obesity and hyperglycemia might explain similar independent negative correlations of ghrelin with central obesity and fasting blood glucose[316].

Although obestatin was initially identified as an appetite suppressant, the role of this ghrelin gene product in metabolism is currently under debate[137,317]. Previous works have showed that postprandial obestatin but not fasting was decreased in diabetic subjects when compared to normal healthy subjects[143,318]. Our study demonstrated that obestatin was elevated in humans with central obesity and individuals with the cluster of the other four MetS risk factors and specifically, a unique pattern was further revealed in our study adopting a 2 x 2 factorial design. Our data illustrated that the level of obestatin was elevated in subjects with central obesity (NRFO and RFO) when compared to the subjects without central obesity (NRFNO and RFNO). These findings suggested that obestatin

demonstrated the interaction of central obesity with the other four MetS risk factors and might play a unique role in energy related disorders. In line with our findings, a recent study revealed significant positive correlations between fasting obestatin and body mass index, low-density lipoprotein cholesterol, and insulin in children and adolescents[319]. Future research is needed to elucidate how the ghrelin gene products exert physiological function in relation to the regulation of energy metabolism in individuals with central obesity and MetS. The roles of ghrelin gene products in metabolism are complex and not yet fully understood. For instance, AG promotes food intake and the release of GH3 while UnAG supports or antagonizes AG via unknown receptors[188,312], whereas obestatin inhibits food intake6 but might not have an effect on body weight[228]. Here, we report a possible role of the ratios of ghrelin gene products in reflecting the interaction of central obesity with the other defined risk factors of MetS. The ratios of ghrelin gene products have been suggested as useful parameters for defining certain energy-related abnormalities including obesity, anorexia nervosa and bulimia nervosa. Guo et al. reported that the ratio of ghrelin/obestatin was significantly higher in overall obese individuals when compared to the control subjects[143]. Monteleone et al. also discovered that the ratio of ghrelin/obestatin was significantly increased in underweight patients with anorexia nervosa but not in symptomatic patients with bulimia nervosa[174]. These previous findings implicated that the ratio of ghrelin/obestatin is probably involved in the energy-related metabolic disorders. Results from our study suggested that central obesity or the cluster of the other four MetS risk factors might probably reflect changes in ghrelin gene expressions, procession of preproghrelin and/or the cleavage of its products, and that the ratios of ghrelin gene

products might affect the overall energy homeostasis[174]. Thus, in essence, our results support the notion that the ratios of ghrelin gene products probably reflect metabolic abnormalities commonly observed in individuals with MetS.

Nesfatin-1 is secreted by several tissues but the precise role of this peptide in energy metabolism remains obscure[320]. In rats, hypothalamic expression of nesfatin-1 was decreased during starvation but its administration was shown to decrease food intake in a dose-dependent manner and reduce body weight[274]. Our results revealed that nesfatin-1 tended to be increased in subjects with the cluster of four or all five MetS risk factors when compared to subjects with only central obesity or no MetS risk factor. As body fuels including glucose and fatty acids are generally higher in obese or MetS individuals, the corresponding higher level of nesfatin-1 might reflect a physiological stimulus to decrease food consumption in these individuals. This study confirmed a previous report indicating a significant decrease in fasting GH in subjects diagnosed with MetS[271]. It has been reported that hypertension and higher body mass increased the clearance of GH by the kidneys after an overnight fast[321]. The decreased GH in central obese subjects when compared to non-central obese subjects in our study might be related to its increased renal clearance. In the current study, GH was decreased in subjects with the cluster of four MetS risk factors when compared to subjects without the cluster of four MetS risk factors and this might also be related to ghrelin secretion as evidenced by their similar associations with the risk factors of MetS. We hypothesized this relationship based on empirical evidence that AG directly stimulates GH release. There is dearth of evidence relating UnAG to GH but both peptides were found to show significant independent associations

with all risk factors of MetS in the present study, suggesting a close link between fasting UnAG and GH. IGF-1 is mainly produced by the liver and has endocrine, paracrine, and autocrine effects[271,304]. Apart from mediating several actions of GH in vivo, such as anabolism, IGF-1 affects other aspects of metabolism. IGF-1 and proinsulin are roughly 50% indistinguishable in their amino acid sequence identity[270]. As a result of the similarity between IGF-1 and proinsulin, IGF-1 is known to bind to insulin receptors, exert insulin-like actions and increase insulin sensitivity in the liver and skeletal muscle of mice[322,323]. We report here that IGF-1 was neither different between subjects with the cluster of four MetS risk factors and subjects without the cluster of four MetS risk factors nor between central obese and non-central obese individuals. Also, there was no correlation between IGF-1 and the cardiometabolic risk factors. Our findings corroborated a previous study demonstrating that obese and control subjects had similar IGF-1 level after an overnight fast[321]. This result might be related to the local production of IGF-1 in virtually all tissues of the body[324], such that its level might remain fairly stable even in the fasting state. Another plausible explanation for the similar IGF-1 level among NRFNO, NRFO, RFNO and RFO groups in this study might be related to the unique regulation of IGF-1 by insulin-like growth factor binding proteins which are known to alter the half-life of the peptide from minutes to hours or by acting as its substrates[325]. By increasing the half-life, IGF-1 probably remains fairly constant despite changes in GH circulation even in the fasting state. The increasing prevalence of central obesity and MetS arouses the need to comprehend the complex physiological processes from which obesity and MetS emerge. The prevalence of coronary arterial disease has been demonstrated to be relatively low in diabetic subjects

without MetS in comparison to diabetic individuals diagnosed with MetS[326]. The interactions of central obesity and the other MetS risk factors as revealed by obestatin, GH and obestatin/ghrelin ratios in the current study suggest that disorders resulting from MetS might be compounded in the presence of central obesity. These novel data provide evidence in support of the current IDF definition of MetS that positions central obesity as the compulsory risk factor, plus two or more of the remaining four risk factors for MetS diagnosis. Since ghrelin gene products, nesfatin-1 and GH levels are altered corresponding to the risk factors of MetS, they might serve as potential targets for further understanding the biochemical pathways related to MetS. Additional researches are warranted to dissect the exact roles of these peptide biomarkers in the progression of MetS and the subsequent chronic diseases such as cardiovascular diseases, type 2 diabetes mellitus and hypertension.

Chapter 4. Ghrelin axis reveals the interacting influence of central obesity and hypertension

(Remark: The findings presented in this chapter have been submitted to *Obesity* - Ugwu, FN, Yu AP, Tam BT, Lee PH, Lai CW, Wong CS and Siu PM (2017). Ghrelin axis reveals the interacting influence of central obesity and hypertension)

4.1. Introduction

Obesity and hypertension, which respectively refer to excess body fat mass and abnormally high blood pressure, are unfavorable conditions that increase morbidity and mortality. The association between hypertension and obesity was suggested in a 26-year follow-up research of the Framingham Heart Study[327]. According to the reports published over three decades ago, obesity, defined as Metropolitan Relative Weight, was identified as a primary independent predictor of cardiovascular diseases[327]. Since the inception of the Framingham Heart Study, the relationship between hypertension and obesity and the mechanisms that lead to obesity-related hypertension have become areas of intensive research. For instance, a joint research by the European Society of Hypertension and the European Society of Cardiology revealed a strong correlation between hypertension and excess body weight in adults, suggesting that the presence of obesity might influence the development of hypertension in adults[40]. Obese children have been shown to have a threefold increase in the risk for hypertension when compared to their lean counterparts and conversely, high blood pressure in children has been strongly linked to increased prevalence of adiposity[328]. Indeed, both hypertension and central obesity are key components of metabolic syndrome which is a combination of cardiometabolic risk factors that predisposes individuals to cardiovascular diseases and type 2 diabetes mellitus[329].

Ghrelin signaling pathway is one of the proposed therapeutic targets for the management of energy homeostasis and cardiovascular function[330]. Ghrelin gene encodes three peptides namely unacylated ghrelin (UnAG), acylated ghrelin (AG) and obestatin. Ghrelin and

obestatin are implicated in several disorders including obesity, type 2 diabetes and cardiovascular diseases[331-333]. Recent reports demonstrated that infusion of AG, but not UnAG, decreased blood pressure in healthy humans[334]. In addition to long-lasting growth hormone (GH) release, intravenous injection of AG caused a stable heart rate, reduced cardiac afterload but increased cardiac output in humans[220], suggesting that AG might play a role in the control of cardiovascular event. The co-administration of ghrelin and obestatin has been reported to exert several antagonistic effects in rats[137]. Obestatin has been shown to be higher in hypertensive rats compared to the controls[245], but a separate investigation indicated that 3 different intravenous obestatin injections in hypertensive rats failed to alter mean blood pressure, heart rate or baroreflex sensitivity compared to the baseline[335]. It is generally acceptable that AG is more potent than UnAG although UnAG represents the predominant form of circulatory ghrelin. A common pitfall of several studies is the lack of distinction between the two forms of ghrelin which may oppose each other depending on the system investigated. For instance, a recent study revealed that fasting ghrelin and obestatin in obese hypertensive subjects were significantly lowered when compared with obese subjects while ghrelin and obestatin were reduced in obese subjects when compared with healthy controls[297]. Although the authors suggested that lowered ghrelin and obestatin might be linked to hypertension and obesity[297], the form of ghrelin was not identified in the study. Therefore, it is not known whether conclusions from the authors were based on AG, UnAG or total ghrelin. Also, it is difficult to describe the contributory role of UnAG or AG from a study reporting that fasting total ghrelin, and total ghrelin/obestatin ratio were significantly lowered in hypertensive adults compared with the controls[336]. AG

mainly causes the direct release of GH from the pituitary gland. It has been reported that GH treatment normalized blood pressure and improved cardiovascular function in rats exposed to adverse prenatal or postnatal conditions[337]. This suggested that a synergistic action between AG and GH might be beneficial in regulating blood pressure. Although GH intervention failed to reverse hyperphagia, GH intervention in children diagnosed with Prader–Willi syndrome was shown to antagonize the progression of obesity, lower blood pressure and improve metabolic profile[338].

It appears the rising prevalence of central obesity parallels that of hypertension as evidenced by several studies. Previously, we reported that approximately 50% and 22% subjects had elevated systolic and diastolic blood pressure, respectively, whereas 48% subjects had central obesity among 3967 subjects screened for metabolic syndrome in Hong Kong[2]. Also, a report of the Third National Health and Nutrition Examination Survey (NHANES) study revealed an increase in elevated blood pressure from approximately 36% and 32% in 1988–1994 to 45% and 41% in 2007–2012 among men and women, respectively[339]. Similarly, results of the NHANES study indicated that among men and women, waist circumference increased from approximately 24% and 38% in 1988–1994 to 43% and 61% in 2007– 2012, respectively[339]. These shocking results indicated that the incidences of hypertension and central obesity might be interrelated.

Both central obesity and hypertension are known to have environmental causes that exceed genetic factors. Furthermore, the most important diet-related factors associated with high blood pressure (hypertension) include overweight and obesity. It has also been documented

that natural remedies for central obesity often correct or minimize the consequences of hypertension. For instance, increased physical activity with a structured exercise program is usually recommended for adults with central obesity, hypertension or central obesity and hypertension. The inability of previous studies to examine the interaction of central obesity and hypertension might have stemmed from several confounding risk factors, especially the remaining 3 MetS risk factors (elevated triglycerides, elevated fasting blood glucose and reduced High-density lipoprotein cholesterol). Therefore, the understanding of the interaction of hypertension and central obesity might be important to further understand the development of cardiovascular diseases and energy-related disorders[2]. In spite of the overwhelming evidence that ghrelin gene products and GH are independently affected by hypertension and central obesity, studies addressing the inter-relationship and interaction of hypertension and obesity on ghrelin gene products and GH are lacking. The present study aimed to examine the interacting influence of hypertension and central obesity on circulatory UnAG, AG, obestatin, total ghrelin, ratios of ghrelin/obestatin, and GH.

4.2. Methods

4.2.1. Subjects

This study was a follow-up investigation examining participants previously screened for metabolic syndrome between November 2010 and August 2013[2]. Female subjects were included in the present study because of the previous findings revealing gender differences in AG, total ghrelin, obestatin and GH[340-343]. Besides, by selecting only female subjects,

the current study attempted to interpret the interaction of hypertension with central obesity without the complications due to gender combination as previously demonstrated[344]. In this study, fasting sera of 387 Hong Kong Chinese female adults aged ranged from 24 to 86 years were retrieved from a total of 1492 archived samples. The included subjects had neither hypertension nor central obesity (n = 105), hypertension but no central obesity (n = 102), central obesity but no hypertension (n = 74) or both hypertension and central obesity (n = 102). Hypertension and central obesity were diagnosed according to American Heart Association and National Cholesterol Education Program (NCEP)-ATP III. Hypertension was diagnosed as systolic or diastolic blood pressure equal to or greater than 140 mmHg or 90 mmHg whereas central obesity was diagnosed as waist circumference exceeding 80 cm[329,345]. All subjects had normal fasting triglycerides, blood glucose and HDL-C based on NCEP-ATP III diagnostic criteria. A 2 x 2 factorial research design was adopted to evaluate the interaction of hypertension with central obesity. Furthermore, data were analyzed by classifying the participants into 4 groups: NHNO – no hypertension no central obesity (n = 105), HNO – with hypertension but no central obesity (n = 102), NHO – without hypertension but with central obesity (n = 74) or HO – with hypertension and central obesity (n = 102). Blood pressure was determined by an electronic blood pressure monitor (Accutorr Plus, Datascope). Waist circumference was measured with an inelastic measuring tape. Sera were separated from fasting venous blood samples obtained after at least 10 hours fast by phlebotomists. All sera samples were aliquoted and stored at -80°C until needed for measurements of UnAG, AG, obestatin and GH. Human research ethics approval was obtained from the human subject ethics subcommittee of the Hong Kong

Polytechnic University (ethics approval number: HSEARS20150203002) and written informed consent was obtained from the subjects prior to commencement of the experiment.

4.2.2. Peptide measurements

UnAG and AG: UnAG and AG ELISA kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RA194063400R and RA194062400R, respectively). The assays were based on a double-antibody sandwich technique. Briefly, the wells of each human UnAG or AG antibody-coated microplate were rinsed before dispensing standards, dilution buffer, samples, quality controls and conjugate solution (blank) into appropriate wells. Plates were covered and incubated for 20 hours at 4°C. Plate contents were discarded, rinsed adequately and blotted on paper towels to remove liquid traces. Substrate solution (Ellman's reagent) was added to each well and the plate was read at 410 nm using a spectrophotometer. The plate was checked periodically every 30 minutes until maximum absorbance was attained. The results were calculated after the average of the blank readings were subtracted from each well.

Obestatin: Human obestatin kits were purchased from RayBiotch, Norcross, USA (EIA-OBS). A biotinylated obestatin peptide was spiked into standards and samples. The samples were then added to appropriate wells where biotinylated obestatin competed with the endogenous obestatin for binding to the anti-obestatin antibody. The plates were incubated for 90 minutes at room temperature. The plates were washed prior to the introduction of horseradish peroxidase-streptavidin which catalyzed a color development

reaction. Each plate was incubated at room temperature for 45 minutes, washed and blotted against a paper towel. The substrate reagent was added to each well, followed by 30 minutes of incubation in the dark. Stop solution was added and each plate was immediately read at 450 nm with a spectrophotometer.

Growth hormone: Human growth hormone kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RCD017R). The assay principle was based on a sandwich type assay. Calibrators, control and specimen samples were added to human antibody-GH-coated microplate wells. Conjugate solution was added to each well, followed by 60 minutes of incubation at room temperature. Wells were washed prior to introduction of substrate solution. Stop solution was added to each well after 15 minutes of incubation. Plates were read immediately using a spectrophotometer at 450 nm.

4.2.3. Statistical analysis

Values are expressed as mean ± standard deviation. The main effects of hypertension and central obesity and the interaction effect of hypertension with central obesity on UnAG, AG, total ghrelin, obestatin, ratios of UnAG/obestatin, AG/obestatin and total ghrelin/obestatin and GH were analyzed by generalized estimating equations (GEE). GEE was adopted as it has less stringent requirement on normality assumption. Statistical differences among the four individual groups were determined by Kruskal-Wallis H Test followed by Dunn-Bonferroni post hoc tests. Statistical significance was accepted at $p <$

0.05. All statistical procedures were conducted using the Statistical Package for the Social Sciences (SPSS) version 22 for Windows.

4.3. Results

4.3.1. Obestatin but not ghrelin or GH revealed the interaction of hypertension with central obesity

No significant interaction effect of hypertension and central obesity was found on UnAG (Figure 4.1A). However, there were significant main effects of hypertension ($p = 0.01$) and central obesity ($p = 0.027$) on UnAG (Figure 4.1A). There was significant difference in UnAG ($p = 0.003$) among the four groups, as revealed by our Kruskal-Wallis H Test (Figure 4.1A). Post hoc analyses showed significant decrease in UnAG in subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity, subjects with only hypertension and subjects with only central obesity ($p = 0.007$, 0.017 and 0.035, respectively) (Figure 4.1A).

No significant interaction effect of hypertension and central obesity was found on AG (Figure 4.1B). There was significant main effect of hypertension on AG ($p = 0.001$). However, there was no main effect of central obesity on AG (Figure 4.1B). There was a significant difference in AG ($p = 0.001$) among the four groups (Figure 4.1B). Our post hoc analyses showed significant decrease in AG in subjects with both hypertension and

central obesity when compared to subjects with only central obesity (p = 0.049), as well as significant decrease in AG in subjects with only hypertension and subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity (p = 0.008 and 0.001, respectively), (Figure 4.1B).

No significant interaction effect of hypertension and central obesity was found on total ghrelin (Figure 4.1C). However, there were significant main effects of hypertension (p = 0.006) and central obesity (p = 0.025) on total ghrelin (Figure 4.1C). There was significant difference in total ghrelin (p = 0.002) among the four groups, as revealed by our Kruskal-Wallis H Test (Figure 4.1C). Post hoc analyses showed significant decrease in total ghrelin in subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity, subjects with only hypertension, and subjects with only central obesity (p = 0.004, 0.015 and 0.025, respectively) (Figure 4.1C).

Significant interaction effect of hypertension and central obesity was found (p = 0.001) on obestatin (Figure 4.1D). There was no main effect of hypertension on obestatin. However, there was significant main effect of central obesity (p = 0.002) on obestatin (Figure 4.1D). Comparing with the subjects with neither hypertension nor central obesity, subjects with only hypertension and subjects with both hypertension and central obesity had no difference in obestatin (p = 0.101 and 0.220, respectively), whereas the subjects with only central obesity had higher obestatin (mean difference = 3.2, p = 0.001). There was significant difference in obestatin (p = 0.001) among the four groups (Figure 4.1D). Further

post hoc analyses showed significant increase in obestatin in subjects with only central obesity when compared to subjects with neither hypertension nor central obesity and subjects with both hypertension and central obesity ($p = 0.001$ and 0.008, respectively), (Figure 4.1D).

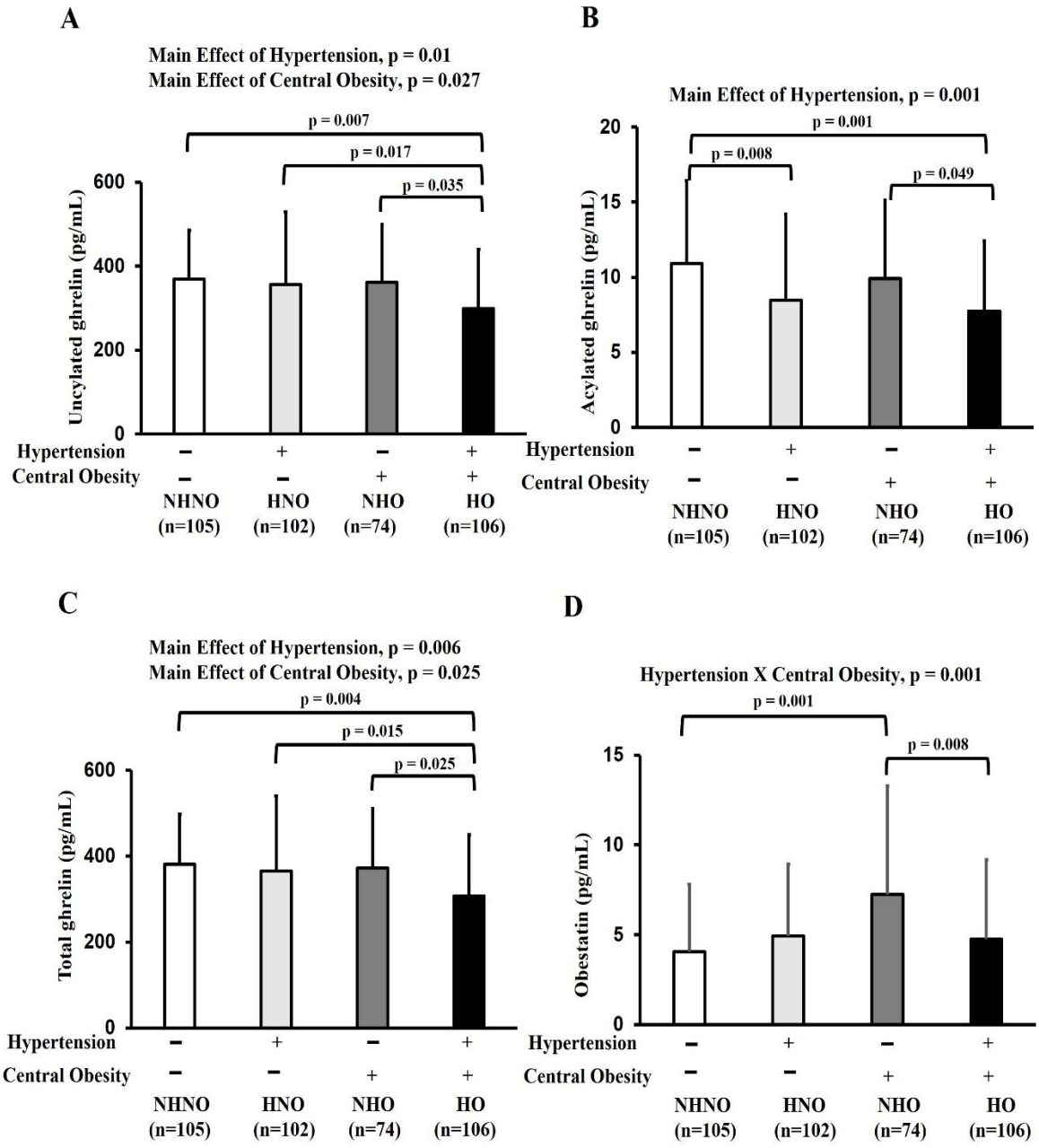

Figure 4. 1 Ghrelin gene products: unacylated ghrelin, acylated ghrelin, total ghrelin and obestatin

There were significant main effects of hypertension and central obesity on unacylated ghrelin and total ghrelin (A, C). There was significant main effect of hypertension on acylated ghrelin (B). Unacylated ghrelin, acylated ghrelin and total ghrelin were decreased in subjects with hypertension and central obesity (A - C). Acylated ghrelin also decreased in subjects with hypertension (B). Obestatin revealed the interaction of hypertension with central obesity (D). Obestatin increased in subjects with only central obesity but decreased in subjects with hypertension and central obesity (D). The main effects of hypertension and central obesity and the interaction of hypertension with central obesity were analyzed with generalized estimating equations. Significance level was set at $p < .05$

NHNO – no hypertension no central obesity; HNO – with hypertension but no central obesity; NHO – without hypertension but with central obesity; HO – with hypertension and central obesity.

4.3.2. Ratios of ghrelin gene products but not GH reflected the interaction of hypertension and central obesity

Significant interaction effect of hypertension and central obesity was found ($p = 0.001$) on UnAG/obestatin ratio (Figure 4.2A). There were significant main effects of hypertension ($p = 0.02$) and central obesity ($p = 0.001$) on UnAG/obestatin ratio (Figure 4.2A). Comparing with the subjects with neither hypertension nor central obesity, subjects with only hypertension and those with both hypertension and central obesity had lower UnAG/obestatin ratios (mean differences = 203.8, 206.7 and $p = 0.002$ and 0.001, respectively), whereas subjects with only central obesity had lower UnAG/obestatin ratio (mean difference = 252, $p = 0.001$) when compared to subjects with neither hypertension nor central obesity (Figure 4.2A). Our Kruskal-Wallis H Test revealed significant difference in UnAG/obestatin ratio ($p = 0.001$) among the four groups (Figure 4.2A). Post hoc analyses showed significant decrease in UnAG/obestatin ratio in subjects with only central obesity and subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity ($p = 0.001$ and 0.006, respectively) (Figure 4.2A).

Significant interaction effect of hypertension and central obesity was found ($p = 0.002$) on AG/obestatin ratio (Figure 4.2B). There was no main effect of hypertension on AG/obestatin ratio. However, there was significant main effect of central obesity ($p = 0.001$) on AG/obestatin ratio (Figure 4.2B). Comparing with the subjects with neither hypertension nor central obesity, those with only hypertension and those with both

hypertension and central obesity had lower AG/obestatin ratios (mean differences = 3.9, 4.4 and p = 0.012 and 0.002, respectively), whereas subjects with only central obesity had much lower AG/obestatin ratio (mean difference = 5.7, p = 0.001) when compared to subjects with neither hypertension nor central obesity (Figure 4.2B). There was significant difference in AG/obestatin ratio (p = 0.001) among the four groups (Figure 4.2B). Post hoc analyses showed significant decrease in AG/obestatin ratio in subjects with only hypertension, subjects with only central obesity and subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity (p = 0.001, 0.001 and 0.001, respectively) (Figure 4.2B).

Significant interaction effect of hypertension and central obesity was found (p = 0.001) on total ghrelin/obestatin ratio (Figure 4.2C). Also, there were significant main effects of hypertension (p = 0.02) and central obesity (p = 0.001) on total ghrelin/obestatin ratio (Figure 4.2C). Comparing with the subjects with neither hypertension nor central obesity, subjects with only hypertension and subjects with both hypertension and central obesity had lower total ghrelin/obestatin ratios (mean differences = 207.7, 211.1 and p = 0.002 and 0.001, respectively) whereas subjects with only central obesity had much lower total ghrelin/obestatin ratio (mean difference = 257.7, p = 0.001) when compared to subjects with neither hypertension nor central obesity (Figure 4.2C). Our Kruskal-Wallis H Test revealed significant difference in total ghrelin/obestatin ratio (p = 0.001) among the four groups (Figure 4.2C). Post hoc analyses showed significant decrease in total ghrelin/obestatin ratio in subjects with only central obesity and subjects with both

hypertension and central obesity when compared to subjects with neither hypertension nor central obesity ($p = 0.001$ and 0.006, respectively) (Figure 4.2C).

No significant interaction effect of hypertension and central obesity was found on GH (Figure 4.2D). However, there were significant main effects of hypertension ($p = 0.001$) and central obesity ($p = 0.001$) on GH (Figure 4.2D). There was significant difference in GH ($p = 0.001$) among the four groups (Figure 4.2D). Further Post hoc analyses showed significant decrease in GH in subjects with both hypertension and central obesity when compared to subjects with neither hypertension nor central obesity, subjects with only hypertension and subjects with only central obesity ($p = 0.001$, 0.003 and 0.018, respectively) (Figure 4.2D).

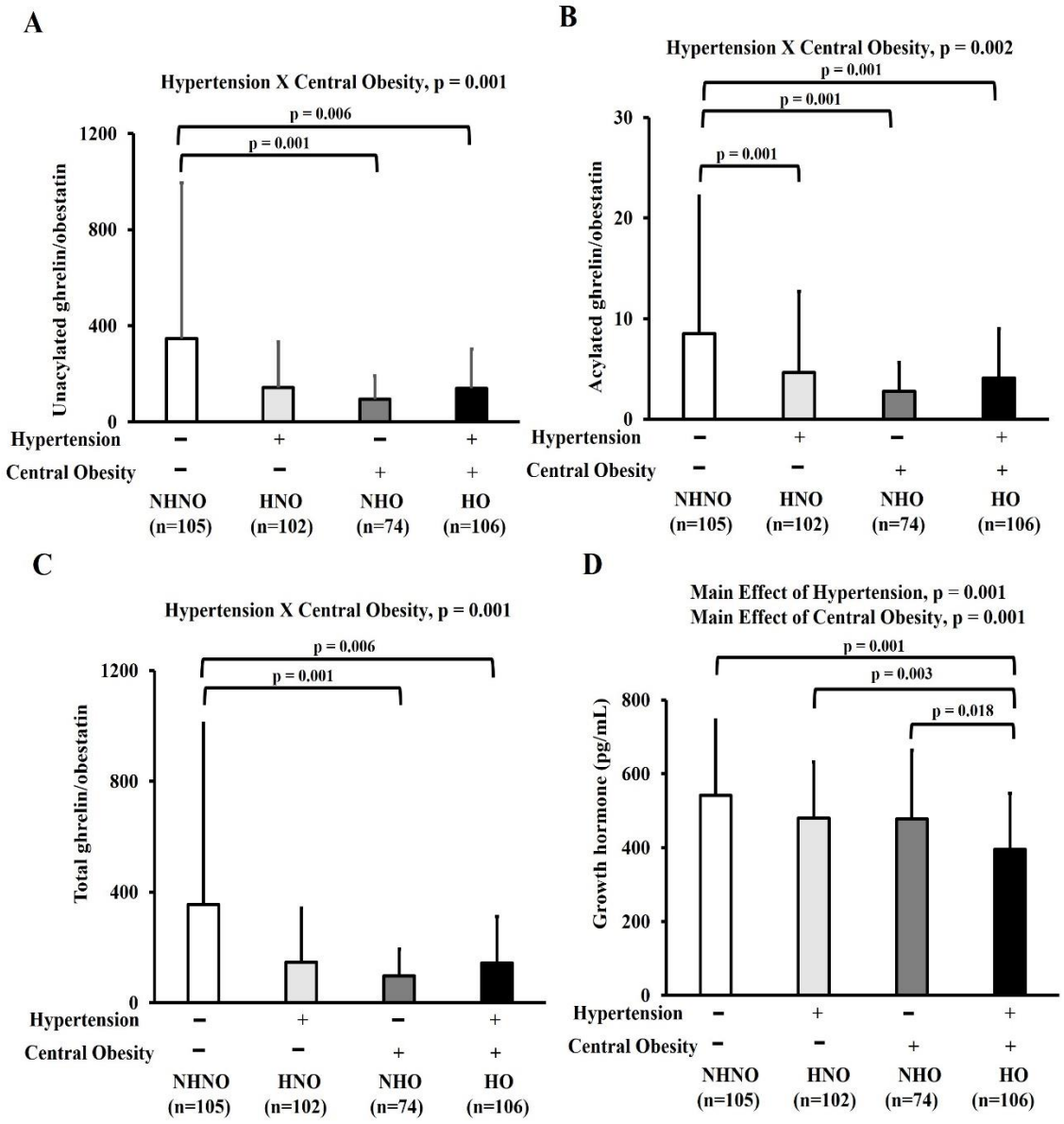

Figure 4.2 Ratios of ghrelin gene products (unacylated ghrelin/obestatin, acylated ghrelin/obestatin and total ghrelin/obestatin) and growth hormone

There was significant interaction of hypertension with central obesity on unacylated ghrelin/obestatin, acylated ghrelin/obestatin and total ghrelin/obestatin ratios (A - C). The ratios of unacylated ghrelin/obestatin and total ghrelin/obestatin were decreased in obese and hypertensive obese subjects (A, C). The ratio of acylated ghrelin/obestatin was decreased in hypertensive, obese and hypertensive obese subjects (B). There were significant main effects of hypertension and central obesity on growth hormone (D). Growth hormone was decreased in subjects with hypertension and central obesity (D). The main effects of hypertension and central obesity and the interaction of hypertension with central obesity were analyzed with generalized estimating equations. Significance level was set at $p < .05$

NHNO – no hypertension no central obesity; HNO – with hypertension but no central obesity; NHO – without hypertension but with central obesity; HO – with hypertension and central obesity.

4.4. Discussion

A cross-sectional survey involving data from NHANES estimated that severe overweight contributed to approximately 60–70% of hypertension in adults, whereas a greater than 20% adiposity existed in 60% of hypertensive adults[346]. Our results revealed that hypertension and central obesity might cohesively influence the circulatory abundance of obestatin and ratios of UnAG/obestatin, AG/obestatin and total ghrelin/obestatin.

Our results are consistent with previous findings showing that hypertensive obese subjects had lowered plasma ghrelin in comparison to normotensive obese or healthy control subjects[297]. It is noteworthy that the form of ghrelin was not specified in the previous investigation. Although UnAG was originally recognized as an inert molecule, recent publications suggested that UnAG might potentiate AG action or exert independent beneficial metabolic effects. For instance, a recent study demonstrated that continuous infusion of AG plus UnAG in healthy subjects decreased systolic and diastolic blood pressure, mean arterial blood pressure, heart rate and body surface temperature, but raised plasma aldosterone level compared to saline-infused control subjects[334]. The beneficial cardiovascular effects of AG plus UnAG were linked to elevated plasma aldosterone but stable renin and catecholamine levels[334]. The decreased UnAG observed in our examined obese subjects in the present study might be mainly attributed to the impact of central obesity. This proposition is in line with a study reporting negative association between UnAG with body mass index in obese individuals and their non-obese counterparts[347]. Several studies have suggested that AG might be involved in the regulation of food intake

and energy homeostasis. Intravenous injection of AG is known to increase food intake among healthy humans[348,349]. In our study, central obese-only subjects did not show decreased level of AG; rather, AG was reduced in hypertensive subjects no matter with or without central obesity, compared to normotensive non-obese control subjects. We speculated that the unaltered AG level in subjects with only central obesity as compared to the control group might be related to the GH-stimulatory effect of AG which promotes lipogenesis.

In the current study, total ghrelin was largely reflected by UnAG which is the predominant form of circulatory ghrelin[350]. Our results revealed that a significant proportion of total ghrelin functions might be attributed to UnAG. It is noteworthy that the similar profiles of UnAG and total ghrelin in our study substantiated findings from a few published studies. For instance, plasma insulin and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) have been demonstrated to correlate negatively with UnAG and total ghrelin but positively associated with AG[347]. Our findings that subjects with both hypertension and central obesity recorded the lowest UnAG, AG and total ghrelin levels among the four individual groups indicated that low ghrelin profile might contribute to pathologic features associated with obesity-related hypertension. It is intriguing that hypertension alone significantly decreased AG but not UnAG or total ghrelin. We speculate that the conversion of UnAG to AG by ghrelin O-acyltransferase enzyme might have been decreased or that AG might have been increasingly degraded to UnAG in hypertensive individuals.

Fasting plasma obestatin has been shown to decrease in hypertensive individuals compared with normotensive control subjects, suggesting that obestatin might play a role in blood pressure regulation[336]. In the current study, the interaction of hypertension with central obesity was significantly manifested by obestatin. In essence, obestatin level was the highest in subjects with only central obesity but lowest in subjects with both hypertension and central obesity. Contrary to our results, obestatin has been shown to be lowered in hypertensive obese patients compared to normotensive obese or healthy control subjects[297]. Our findings might be related to the pancreatic effects of obestatin as demonstrated in a few animal studies. Obestatin infusion has been shown to significantly increase the pancreas of newborn rats compared to the control group[351]. Also, obestatin has been demonstrated to mitigate pancreatic weight reduction, β-cell destruction, downregulation of genes that regulate β-cell function and hyperglycemia but increased plasma and pancreatic insulin level in streptozotocin-induced newborn rats in comparison to control rats[351]. At lower obestatin concentrations (5.5 nM and 9 nM), glucose-perfused rat pancreas stimulated insulin secretion in a fashion resembling the ingestion of a carbohydrate-rich meal. In contradistinction, a high obestatin concentration (10 nM) hampered the insulin response elicited by the glucose stimulus[243]. The authors underscored the importance of evaluating a wide range of obestatin doses which might reflect the cause of discrepancies in studies addressing the effect of obestatin on feeding behavior[243]. It is intriguing that obestatin level was similar in non-hypertensive non-obese and hypertensive obese subjects. Results from our study indicated that hypertension and central obesity might have exerted opposing influences on obestatin. Furthermore, our results suggested that in hypertensive

obese subjects, obestatin might act as a compensatory beneficial molecule which might partially maintain metabolism or homeostasis similar to non-hypertensive non-obese individuals. Clearly, further studies are warranted for further elucidating the differential physiological role of obestatin in hypertension and central obesity.

In the current study, significant interaction of hypertension with central obesity was demonstrated by UnAG/obestatin, AG/obestatin and total ghrelin/obestatin ratios. These results indicated that alteration of all three ghrelin gene products might play a role in hypertension and central obesity. According to our results, higher concentration of ghrelin or lower obestatin concentration might reflect healthy cardiovascular and metabolic health. Our findings corroborate a previous study reporting a decreased total ghrelin/obestatin ratio in obese women as compared to normal weight women[352]. Interestingly, total ghrelin/obestatin ratio has been demonstrated to correlate negatively with body mass index, waist circumference, waist/hip ratio and insulin[352]. In another study, normotensive obese subjects displayed negative correlation between ghrelin/obestatin ratio and fasting insulin[297]. More recently, AG/obestatin ratio was shown to be significantly lower in obese children and adolescents compared to their normal-weight counterparts[319]. With regards to hypertension, total ghrelin/obestatin ratio was shown to be significantly lowered in hypertensive subjects compared with normotensive subjects[336]. Our study synthesized previous independent observations of ghrelin/obestatin ratios in hypertension and central obesity by evaluating ghrelin/obestatin ratios in non-hypertensive non-obese, hypertensive, obese and hypertensive obese subjects. Our results suggested that the combination of hypertension and central obesity might decrease ghrelin/obestatin ratios and that alteration

in ghrelin/obestatin ratios might be associated with pathological conditions arising from hypertension and central obesity.

AG stimulates the release of GH from the anterior pituitary[139]. Although GH intervention has been demonstrated to restore blood pressure in diet-induced obese hypertensive Wistar rats compared to standard diet-fed control rats[353], no study has investigated the interaction of hypertension and central obesity on circulatory GH. To the best of our knowledge, the current study is the first to examine the interacting influence of hypertension and central obesity on GH. Our results revealed that GH was independently decreased by hypertension and central obesity. Among the four individual groups, hypertensive obese and normotensive non-obese subjects recorded the lowest and highest GH levels respectively. A striking finding in our study was the similar profiles of GH, UnAG and total ghrelin that were significantly decreased in hypertensive obese subjects compared to normotensive non-obese subjects. AG but not UnAG is the only form of ghrelin that has been proven to physiologically stimulate the release of GH from the anterior pituitary. However, the current study did not observe identical AG and GH profiles, as AG was decreased in hypertensive subjects with or without central obesity as compared to non-hypertensive non-obese subjects. It has been reported that spontaneous and induced GH secretion was decreased in obesity[354], whereas GH replacement in GH-deficient adults resulted in a reduction of central obesity[355]. In the present study, the lower GH level in obese individuals compared to normotensive non-obese subjects might be related to a reduced half-life, frequency and daily production rate of GH as previously reported[354]. Although the signaling molecules downstream of UnAG remain unidentified, our results suggest that

UnAG might indirectly affect GH via unknown signaling pathways. Additionally, a possible explanation for GH pattern which requires further investigation might be related to the influences of other hormones that regulate GH including growth hormone releasing hormone and growth hormone inhibiting hormone.

Hypertension and obesity often present salient clinical features which could compromise health if they are not well-managed. Obesity-related hypertension is stimulating a great deal of interest because understanding the signaling pathways or associated mechanisms could provide important insights in the development of new strategies for the management of two leading cardiovascular epidemics: hypertension and obesity. The current study adds to the previous findings through detailed analysis of ghrelin gene peptides and GH in individuals with neither hypertension nor central obesity as well as individuals with only central obesity, hypertension or both central obesity and hypertension. Our findings that the interacting influence of hypertension with central obesity was revealed by obestatin and the ratios of ghrelin gene products indicated that ghrelin gene products might serve as potential targets for further understanding the biochemical pathways related to cardiovascular and energy-related disorders. Future research involving male subjects are recommended to examine the suspected gender difference in the interaction of hypertension with central obesity. In conclusion, our findings illustrate the disturbances of circulatory ghrelin gene products and GH in subjects with hypertension and/or central obesity which might play a role in the regulation of blood pressure and control of body fat mass.

Chapter 5. Yoga training modulates ghrelin in central obese older adults with metabolic syndrome

(Remark: The findings presented in this chapter have been submitted to *Medicine & Science in Sports & Exercise* - Ugwu, FN, Yu AP, Tam BT, Lee PH, Lai CW, Wong CS and Siu PM (2017). Yoga training modulates ghrelin in central obese older adults with metabolic syndrome)

5.1. Introduction

Sedentary lifestyle and lack of adequate rest might contribute to the development of metabolic syndrome (MetS), which is a cluster of risk factors linked to non-communicable chronic diseases including cardiovascular disease, type 2 diabetes and stroke[356]. MetS is generally diagnosed with the presence of a combination of cardiometabolic risk factors including central obesity, high blood pressure, high fasting blood glucose, high fasting triglycerides, and low HDL-C, with margins proposed by several professional associations such as NCEP-ATP III and World Health Organization[357,358]. The summary of a recent report of studies from 15 countries in the Asia-Pacific region revealed that the prevalence of MetS is on the rise, with approximately 20% of the Asian adult population being affected by MetS[359]. In addition, we have recently reported that the prevalence of MetS in Hong Kong adults is approximately 27%[289]. Under the current terminology, MetS is not classified as a disease but the body's normal physiological systems and functions might have been progressively impaired even without the occurrence of distinct symptoms from the MetS risk factors. Therefore, strategies must be formulated to curb further increase in MetS and prevent the morbidity and mortality associated with MetS. With the current epidemic of obesity attributed to poor lifestyle habit, lifestyle modification is necessary to intercept the progression of MetS. An epidemiological study demonstrated that modest leisure-time with physical activity decreased the prevalence of MetS in over 3,000 individuals, whereas frequent intensive exercise led to a greater decrease in the prevalence of MetS[360]. The 2009 joint interim statement of the International Diabetes Federation Task Force on

Epidemiology and Prevention, National Heart, Lung, and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society and International Association for the Study of Obesity, recommends the adoption of waist measurement as a useful preliminary screening tool for MetS[29].

Recent investigations have led to identification of abnormal peptide profile of ghrelin gene products, growth hormone (GH), and insulin in subjects with MetS[361,362]. Different aspects of metabolism are complexly regulated by ghrelin gene products including unacylated ghrelin (UnAG), acylated ghrelin (AG), and obestatin[363]. Subjects with MetS are known to have lower AG level compared to control subjects and the level of AG is inversely related to the severity of MetS[364,365]. In addition, when compared to healthy controls, the level of AG has been reported to be lower in individuals with central obesity, insulin resistance and high blood pressure, which are all components of MetS[366-368]. Furthermore, the intrinsic interplay between AG and obestatin is implicated in several energy-related disorders such as obesity and MetS. Obese women have been demonstrated to have a higher plasma obestatin level but lower plasma ghrelin level, suggesting that the alteration of these peptides might be associated with obesity and MetS[352]. Although AG directly causes the release of GH from the anterior pituitary gland, exercise has been demonstrated to be a potent stimulus for GH secretion, which leads to lipolysis[369]. Low GH level is associated with obesity[370], suggesting that low GH level might be indirectly involved in the development of central obesity and MetS by causing a decrease in the rate of lipolysis.

Yoga comprises physical, mental and spiritual practices, which aim at uniting individual alertness with universal consciousness[371]. Yoga has been recommended as a cost effective tool for the improvement of metabolic disorders and, like other exercises, it causes minimal or no adverse side effects[372]. Among the variety of yoga practice streams, Hatha yoga is popularly practiced as it balances postures through physical and mental strength building exercises[373]. A few studies have examined the beneficial effects of yoga on health outcomes. Participation in an 8-week Hatha yoga class has been shown to result in reduction in perceived levels of anxiety in women who suffer from anxiety disorders[374]. In contradistinction, a recent study reported that adults with MetS achieved greater reductions in waist circumference and MetS z-score after 12-week of Hatha yoga training compared to MetS control subjects without yoga intervention[373]. An 8-week yoga intervention has been shown to reduce insulin level and insulin resistance based on the measurement of homeostatic model assessment (HOMA) in apparently healthy subjects compared to their pre-intervention baseline values[375]. Although a few studies have investigated the acute effect of yoga on HOMA indices, there is still a dearth of research on the effects of prolonged yoga intervention on HOMA indices in subjects with MetS.

Yoga exercise is professed to have health promoting effects by adherents but the overall effects of yoga on metabolic peptides remain largely unclear. Moreover, studies with prolonged duration of yoga exercise, particularly in the older population are lacking. Recently, we investigated the effects of 1-year yoga training on cardiometabolic risk factors in adults with MetS[289]. We demonstrated that MetS adults who participated in 1-year yoga program showed significant decreases in waist circumference and the number of

MetS risk factors compared to MetS adults without receiving yoga intervention[289].This study further investigated the effect of 1-year yoga training on metabolic peptides including UnAG, AG obestatin, GH and insulin as well as β-cell function and insulin resistance using HOMA model in the MetS older adults with central obesity.

5.2. Methods

5.2.1. Subject and study design

This sub-study examined MetS subjects who participated in 1-year yoga intervention program or served as non-exercise control[289]. In our previous published study, we observed that 1-year yoga training reduced waist circumference but not the other MetS risk factors[289], suggesting that the effect of yoga might have been diluted by the vague combination of MetS risk factors with or without central obesity. To clarify the effect of 1-year yoga training on metabolic peptides including UnAG, AG obestatin, GH and insulin, the current investigation focused on MetS subjects with central obesity as a necessary risk factor among the MetS risk factors including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose and reduced HDL-C, as diagnosed by NCEP-ATP III prior to the commencement of the experiment. Our choice of MetS subjects with central obesity was also supported by findings that central obesity represents the most prevalent correlate of MetS[376] and reports by International Diabetes Federation that central obesity is a necessary criterion to identify patients with MetS[34]. In this study, fasting blood glucose data and sera samples of 79 MetS subjects with central obesity plus at least 2 of

the remaining 4 MetS risk factors including elevated blood pressure, elevated triglycerides, elevated fasting blood glucose and reduced HDL-C, as diagnosed by NCEP-ATP III (yoga, n=39; control, n=40) before and after 1-year of the experimental period were retrieved for analysis. Sera of the subjects before and after 1-year yoga intervention were analyzed by Enzyme Linked Immunosorbent Assay (ELISA) for UnAG, AG, GH and insulin. All the experimental procedures were approved by The Hong Kong Polytechnic University (ethics approval number: HSEARS20160810001).

5.2.2. Yoga intervention

The yoga intervention adopted in this study has been previously described[289]. Briefly, a computer program was employed to randomly assign all subjects into either a control or a yoga group. Monthly health status questionnaires and telephone calls were employed to monitor control subjects throughout the study whereas subjects in the yoga group were assigned to 1-year of yoga exercise training. The yoga training was conducted in small group (~10 subjects in each group) led by certified yoga instructors and performed 3 times weekly for 1 year. Each session lasted approximately 1 hour and consisted of 10-min of warm-up, 40-min of Hatha yoga practice, and 10-min of breathing exercise and relaxation cool-down. Only subjects in the yoga group who completed at least 70% attendance to the training program were included in the study[289].

5.2.3. MetS parameters and peptide determination

Subjects' sera samples and fasting blood glucose data before and after the 1-year experimental period were retrieved for the present study's analysis. The sera were used for the measurement of UnAG, AG, obestatin, GH and insulin. The measured insulin and the retrieved fasting blood glucose data were used for HOMA index calculations. All peptides were determined using commercially available ELISA kits by following the manufacturers' instructions.

UnAG and AG: UnAG and AG kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RA194063400R and RA194062400R, respectively). The assays were based on a double-antibody sandwich technique. The wells of each human UnAG or AG antibody-coated microplate were rinsed before dispensing standards, dilution buffer, samples, quality controls and conjugate solution (blank) into appropriate wells. Plates were covered and incubated for 20 hours at 4°C. Plate contents were discarded, rinsed adequately and blotted on paper towels to remove liquid traces. Substrate solution (Ellman's reagent) was added to each well and the plate was read at 410 nm using a spectrophotometer. The plate was checked periodically every 30 minutes until maximum absorbance was attained. The results were calculated after the average of the blank readings were subtracted from each well.

Obestatin: Human obestatin kits were purchased from RayBiotch, Norcross, USA (EIA-OBS). A biotinylated obestatin peptide was spiked into standards and samples. The samples were then added to appropriate wells where biotinylated obestatin competed with

the endogenous obestatin for binding to the anti-obestatin antibody. The plates were incubated for 90 minutes at room temperature. The plates were washed prior to the introduction of horseradish peroxidase-streptavidin which catalyzed a color development reaction. Each plate was incubated at room temperature for 45 minutes, washed and blotted against a paper towel. The substrate reagent was added to each well, followed by 30 minutes of incubation in the dark. Stop solution was added and each plate was immediately read at 450 nm with a spectrophotometer.

Growth hormone: Human growth hormone kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RCD017R). The assay principle was based on a sandwich type assay. Calibrators, control and specimen samples were added to human antibody-GH-coated microplate wells. Conjugate solution was added to each well, followed by 60 minutes of incubation at room temperature. Wells were washed prior to introduction of substrate solution. Stop solution was added to each well after 15 minutes of incubation. Plates were read immediately using a spectrophotometer at 450 nm.

Insulin: Human insulin kits were purchased from BioVendor – Laboratiorni medicina a.s., Karasek, Czech Republic (RA194062400R). The principle was based on a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microplates. Monoclonal antibodies directed against distinct epitopes of insulin were introduced into the wells of each microplate. Calibrators, control and specimen samples were introduced to react with the capture monoclonal antibody (MAb 1) coated on the well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After a 30-minute

incubation period allowing the formation of a sandwich: coated MAb 1-human insulin-MAb 2-HRP, each microplate was washed to remove unbound enzyme labelled antibody. Chromogenic solution was added to the wells, followed by 15 minutes of incubation. The reaction was terminated with the addition of stop solution and the microplate was then read at 450 nm (reference filter 630).

Beta cell function and insulin resistance: HOMA represents a computer-based model used to estimate beta-cell function and insulin resistance[377]. All HOMA indices were evaluated using fasting glucose and insulin. Since HOMA1, the first model, is limited in its functions, HOMA2, the current model, which allows for wider applications, even in hyperglycemic subjects was adopted in this study[378]. HOMA-%B2, HOMA-%S2 HOMA-IR2 were calculated using the HOMA calculator (https://www.dtu.ox.ac.uk/homacalculator/) whereas disposition index was calculated from multiplication of (HOMA-%S2)/100 by (HOMA-%B2)/100. HOMA-%B2, HOMA-%S2 and HOMA-IR2 represent β-cell function, sensitivity and insulin resistance, respectively[379].

5.2.4. Statistical analysis

Values are expressed as mean ± SD. Normality of the baseline data were determined by Shapiro-Wilk Test. Baseline parameters of subjects in yoga and control groups including UnAG, AG, obestatin, GH, insulin, HOMA-%B2, HOMA-%S2 HOMA-IR2 and disposition index were analyzed by Mann-Whitney U-Test. The number of males and

females in both groups were compared by Chi-Square Test. The main intervention and time effects and their interaction effect on UnAG, AG, obestatin, GH, insulin, HOMA-%B2, HOMA-%S2, HOMA-IR2 and disposition index were analyzed by generalized estimating equations (GEE). GEE was adopted as it is more robust on normality assumption. Statistical significance was accepted at $p < 0.05$. All statistical procedures were conducted using the Statistical Package for the Social Sciences (SPSS) version 22 for Windows.

5.3. Results

The baseline parameters of subjects in intervention and control groups including UnAG, AG, total ghrelin, obestatin, GH, insulin, HOMA-%B2, HOMA-%S2, HOMA-IR2, and disposition index are depicted in Table 1. There was no significant difference in the baseline characteristics between intervention and control groups (Table 1).

Table 5.1 Baseline peptide levels and HOMA indices in MetS control and yoga subjects

	Control group (n = 40)	**Yoga group (n = 39)**	**P-value**
Gender	8 male, 32 female	7 male, 32 female	0.955
Age	57.7 ± 8.6	58.8 ± 8.4	0.569
Unacylated ghrelin (pg/mL)	311.6 ± 201.2	258.7 ± 120.2	0.349
Acylated ghrelin (pg/mL)	7.5 ± 7.1	6.6 ± 4.7	0.627
Obestatin (pg/mL)	1.1 ± 0.7	1.1 ± 0.9	0.988
Total ghrelin (pg/mL)	319.1 ± 199.9	265.3 ± 119.6	0.317
Growth hormone (ng/mL)	15.8 ±4.7	18.5 ± 6.1	0.104
Insulin (pmol/L)	134.0 ± 32.7	132.6 ± 35.9	0.688
HOMA-%B2	153.0 ± 42.1	143.7 ± 44.9	0.814
HOMA-%S2	42.1 ±9.7	44.3 ± 18.5	0.483
HOMA-IR2	2.5 ± 0.6	2.5 ± 0.7	0.483
Disposition index	0.6 ± 0.1	0.6 ± 0.1	0.259

There was no significant difference in the baseline unacylated ghrelin, acylated ghrelin, total ghrelin, obestatin, growth hormone, insulin, HOMA-%B2, HOMA-%S2, HOMA-IR2 and disposition index between control and yoga groups. Sex was analyzed by Chi-Square Test whereas other parameters were analyzed by Mann-Whitney U-Test. MetS, Metabolic syndrome; HOMA, Homeostatic model assessment; HOMA-%B2, HOMA-%S2 and HOMA-IR2 = β-cell function, sensitivity and insulin resistance, respectively. Significance level was set at $p < .05$.

5.3.1. Yoga training decreased AG and obestatin and tended to increased UnAG and total ghrelin

The changes in ghrelin gene products measured at 1-year relative to baseline (pre) in yoga and control groups are shown in Figure 1. There was a significant interaction ($p = 0.022$) of intervention and time on UnAG (Figure 5.1A). UnAG was higher, but marginally insignificant in the intervention group (mean difference = 66.7, $p = 0.071$) compared to the control group after the 1-year experimental period. Subjects in the control group had lower UnAG at the end of 1-year compared to the baseline (mean difference = 82.8, $p = 0.019$). However, there was no difference in UnAG between intervention and control groups after 1-year when compared to their baseline (Figure 5.1A). There was no significant interaction of intervention and time on AG (Figure 5.1B). Also, there was no effect of time on AG level. However, there was a significant effect of intervention ($p = 0.044$) on AG. AG was lower in the intervention group (mean difference = 2.5, $p = 0.016$) when compared to control group at the completion of the 1-year experimental period. In addition, AG was lower (mean difference = 2.5, $p = 0.019$) in intervention group at the end of the experimental period when compared to baseline. AG was not different in control group before and after the experiment (Figure 5.1B). There was a significant interaction ($p = 0.023$) of intervention and time on total ghrelin (Figure 5.1C). Total ghrelin was higher, but marginally insignificant in the intervention group (mean difference = 64.2, $p = 0.082$) compared to control group after the 1-year experimental period. Subjects in control group had lower total ghrelin at the end of 1-year compared to baseline (mean difference = 83.4, $p = 0.018$). However, there was no difference in total ghrelin between intervention and

control groups after the 1-year experimental period when compared to their respective baseline measurements (Figure 5.1C). There was a significant interaction ($p = 0.026$) of intervention and time on obestatin (Figure 5.1D). There was a significant effect of intervention on obestatin ($p = 0.045$). Obestatin was lower in intervention group when compared to control group (mean difference = 0.6, $p = 0.003$) at the end of the 1-year experimental period. Obestatin tended to be lower in intervention group after 1-year (mean difference = 0.3, $p = 0.088$) compared to the baseline. Obestatin was not different in control group before and after the experiment (Figure 5.1D).

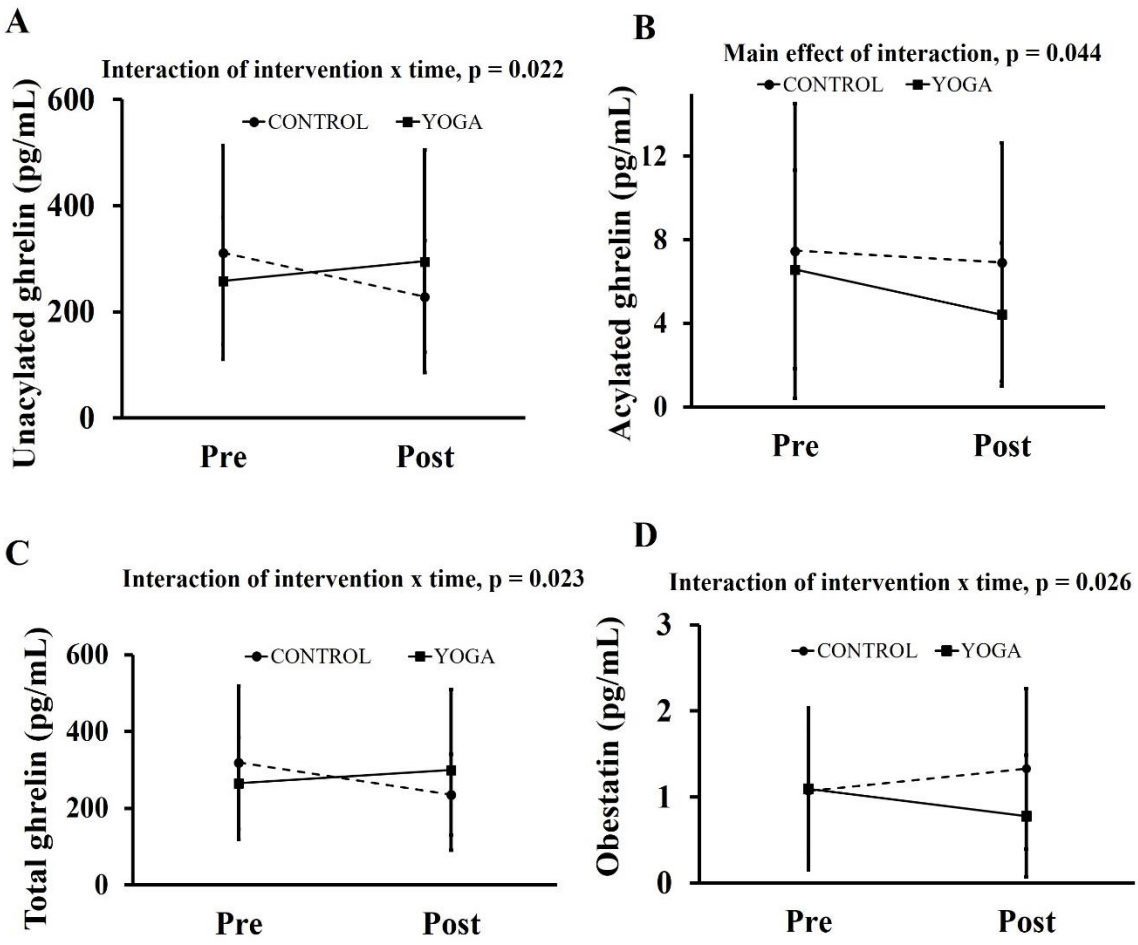

Figure 5. 1 Ghrelin gene products in MetS control and yoga subjects

There was a significant interaction of group and time on unacylated ghrelin, total ghrelin and obestatin (A, C - D). Unacylated ghrelin and total ghrelin tended to be higher in yoga group but obestatin was decreased compared to control group at the end of the 1-year experimental period (A, C - D). Main effect of group on AG was significant and AG was lower in yoga group compared to control group at the end of the 1-year experimental period (B). The main effect of group, the main effect of time and the interaction of group with

time on unacylated ghrelin, acylated ghrelin and obestatin were analyzed by generalized estimating equations (GEE). MetS, Metabolic syndrome; Pre and Post, Before and after the 1-year experimental period respectively. Statistical significance was accepted at $p < 0.05$.

5.3.2. Yoga training increased GH but not insulin

There was a significant interaction of intervention and time on GH ($p = 0.037$) (Figure 5.2A). No time effect on GH was found, whereas a significant main effect of intervention on GH was observed ($p = 0.001$). GH was higher in intervention group at the end of the 1-year experimental period (mean difference = 7.2, $p = 0.001$) when compared to control group. Also, GH was higher in intervention group (mean difference = 4, $p = 0.022$) at the end of the experimental period compared to the baseline. The level of GH did not differ in control group before and after the experiment (Figure 5.2A). There was no interaction of intervention and time on insulin (Figure 5.2B). Neither the time nor intervention effect on insulin level was found (Figure 5.2B).

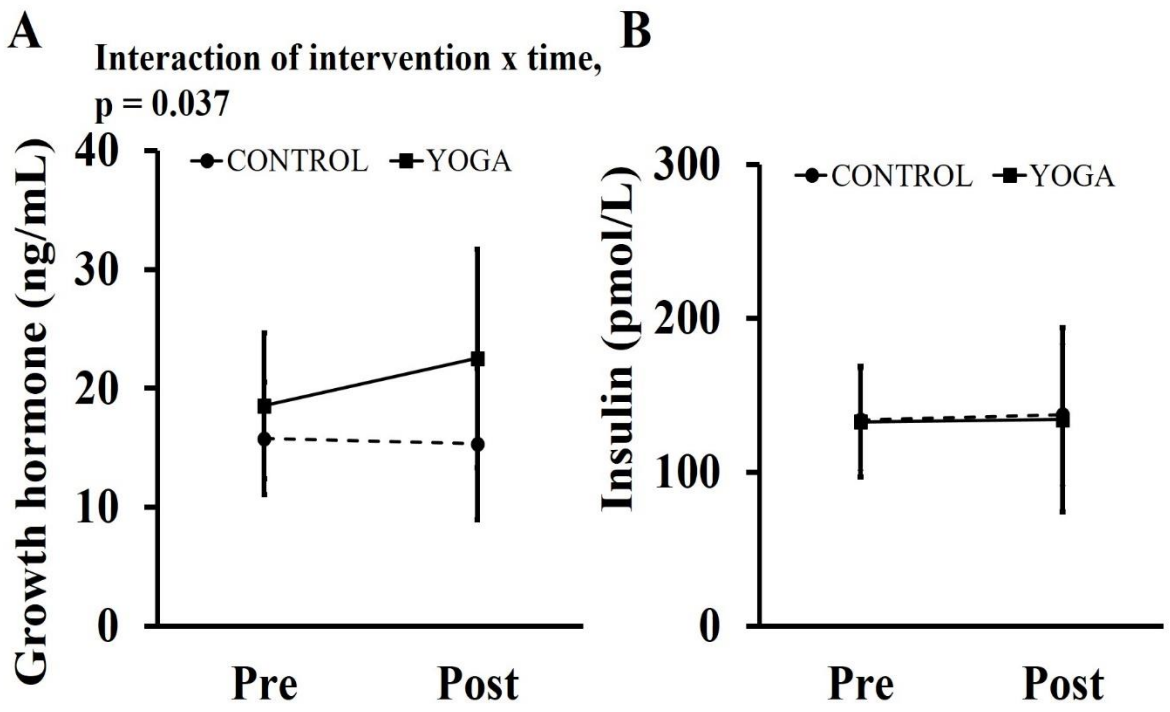

Figure 5. 2 Growth hormone and insulin in MetS control and yoga subjects

There was a significant interaction of group and time on growth hormone (A). Growth hormone was higher in yoga group compared to control group at the end of the 1-year experimental period (A). There was no change in insulin in yoga and control groups at the end of the 1-year experimental period (B). The main effect of group, the main effect of time and the interaction of group with time on growth hormone and insulin were analyzed by generalized estimating equations (GEE). MetS, Metabolic syndrome; Pre and Post, Before and after the 1-year experimental period respectively. Statistical significance was accepted at $p < 0.05$.

5.3.3. Yoga training had no effect on β-cell function and insulin resistance

There was no significant interaction of intervention and time on HOMA-%B2 (Figure 5.3A). Neither the main effect of time nor intervention in HOMA-%B2 was observed (Figure 5.3A). There was no interaction of intervention and time on HOMA-%S2 (Figure 5.3B). Main effect of time in HOMA-%S2 was not found. The main effect of intervention on HOMA-%S2 was marginally insignificant ($p = 0.061$). HOMA-%S2 was higher, but marginally insignificant in intervention group (mean difference = 11.7, $p = 0.078$) at the end of the 1-year experimental period when compared to control group. There was no difference in HOMA-%S2 between both groups compared to their baseline measurements (Figure 5.3B). There was no interaction of intervention and time on HOMA-IR2 (Figure 5.3C). Neither the main effect of intervention nor time in HOMA-IR2 was found (Figure 5.3C). There was no interaction of intervention and time on disposition index (Figure 5.3D). Neither the main effect of intervention nor time in disposition index was found (Figure 5.3D).

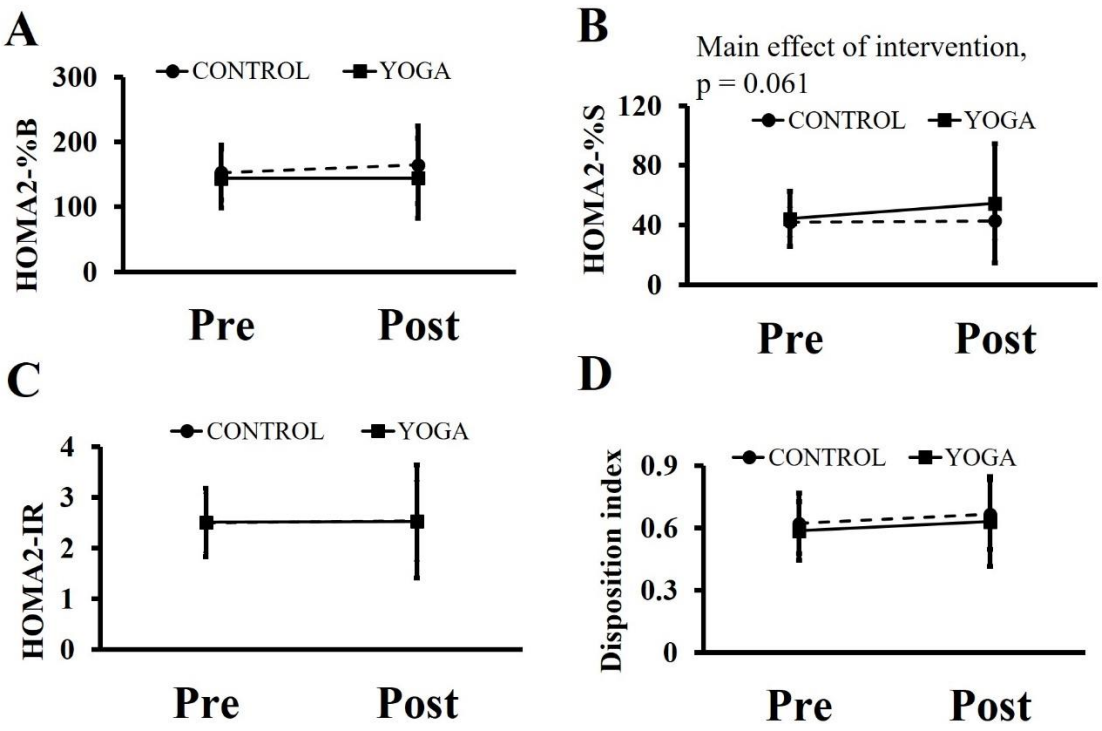

Figure 5. 3 HOMA indices in MetS control and yoga subjects

There was no significant interaction of group and time or main effect of group or time on HOMA-%B2, HOMA-%S2, HOMA-IR2 and disposition index (A - D). HOMA-%S2 tended to increase in yoga group compared to control group at the end of the 1-year experimental period (B). The main effect of group, the main effect of time and the interaction of group with time on HOMA-%B2, HOMA-%S2, HOMA-IR2 and disposition index were analyzed by generalized estimating equations (GEE). MetS, Metabolic syndrome; Pre and Post, Before and after the 1-year experimental period respectively. HOMA, Homeostatic model assessment; HOMA-%B2, HOMA-%S2 and HOMA-IR2 = β-cell function, sensitivity and insulin resistance, respectively. Statistical significance was accepted at $p < 0.05$.

5.4. Discussion

In the current study, we investigated the effects of 1-year yoga training on metabolism-related peptides including GH, insulin and ghrelin gene products namely UnAG, AG, total ghrelin and obestatin in MetS adults with central obesity. β-cell function and insulin resistance were also evaluated using mathematically derived HOMA formulae. Our results revealed that there was a significant interaction of intervention and time on UnAG, total ghrelin, obestatin and GH, but not AG and insulin. UnAG and total ghrelin tended to be higher, GH was higher, and obestatin was lower in intervention group compared to control group after the 1-year experimental period. AG was lower in intervention group when compared to control group whereas insulin, HOMA indices and disposition index were unaltered at the end of the 1-year experimental period. Our data demonstrated that prolonged yoga training modulates the abundances of the metabolism- and appetite-associated peptides in the circulation.

We have previously reported that waist circumference was significantly reduced at the end of the 1-year yoga intervention[289]. UnAG has been reported to be increased but AG showed no change in obese women after 8-week of mild running exercise when compared to control subjects and the changes in UnAG and AG were associated with significant weight loss in the exercise group compared to control group[380]. In another study, 12-week of combined walking and rubber band exercises was shown to increase UnAG by over 100%, whereas AG concentration was unaltered in overweight boys aged 11 years in comparison to control group[381]. Contrary to these studies reporting no change in AG after running and

combined walking and rubber band exercises, the present study found a decrease in AG in the intervention group relative to control group at the end of the 1-year experimental period. The decrease in circulatory AG suggests that AG might play a crucial role in the management of disorders associated with MetS. Evidence for this hypothesis was drawn from published reports indicating that obstruction of endogenous ghrelin signaling in the brain significantly decreased spontaneous food intake and body weight[382,383].

A key strength of the current study is the evaluation of both forms of ghrelin - UnAG and AG. It has been suggested that the reduction of splanchnic blood flow during exercise decrease gastrointestinal perfusion, leading to alteration in total ghrelin/AG ratio[384]. Indeed, our results indicated that all three ghrelin gene products were altered after 1-year of yoga training, with UnAG and total ghrelin tended to be increased but AG and obestatin decreased. The increase in total ghrelin in the current study corroborates previous findings that 1-year of treadmill walking and stationary bicycling increased total ghrelin in overweight women aged 50 – 75 years compared to overweight control women who participated only in stretching exercise[385]. Our findings suggest that total ghrelin largely reflects circulatory UnAG as evidenced by similar concentrations and changing patterns in both groups before and after the experimental period. This argument is supplemented by previous studies showing that fasting UnAG is approximately 13 - 17 times higher than AG in obese and normal subjects[350] and UnAG has a half-life 3 times longer than AG[386]. The increase in UnAG in intervention group at the end of 1-year might indicate the potential of UnAG in restoring metabolic homeostasis in MetS subjects since fasting UnAG is significantly higher in normal healthy subjects when compared to individuals

with MetS risk factor such as central obesity[350]. It has become increasingly clear that UnAG also plays crucial roles in metabolism and it is probable that some conclusions from the past studies that solely examined total ghrelin might have failed to identify the independent effects of the distinct forms of ghrelin. In a recent 2-week clinical trial, single daily injection of AZP-531, an UnAG agonist, was demonstrated to decrease the mean body weight from baseline in obese subjects[387]. We are tempted to infer that the decrease in UnAG and total ghrelin in control group might partly contribute to the observed increase in waist circumference reported in our previous study[289]. Although the mechanisms by which low UnAG increases waist circumference is largely unknown, we speculate that the maintenance of a high circulatory UnAG might prevent the development of central obesity.

Hedayati et al.[388] demonstrated that 4-week of high-intensity resistance training significantly decreased obestatin in adult females compared to healthy controls. These findings stressed that obestatin regulation might be dependent on health condition and the intensity and/or duration of the exercise practiced by the subjects. In the present study, 1-year yoga training was found to reduce obestatin in adults with MetS. Our result is closely related to a study showing that obestatin level was higher in obese women compared to normal-weighed women[352]. Thus, our findings that 1-year of regular yoga training increased the level of UnAG but decreased AG and obestatin substantiate the previous findings that implicated the role of ghrelin gene products in the progression of metabolic disorders. Although complete proof that illuminate the exact mechanisms by which yoga exercise alters the concentrations of ghrelin gene products is lacking, our study affirms the theory that disproportion of circulatory ghrelin gene products might be related to the

pathophysiology of MetS and therapies aimed at normalizing MetS risk factors might function in part by restoring the levels of ghrelin gene products. Further studies are warranted to delineate the mechanisms by which yoga exercise alters the production, secretion and/or breakdown turnover of ghrelin gene products.

The actions of GH are diverse and complex, affecting virtually all tissues of the body. Although AG directly leads to the release of GH, there was a significant increase in GH but not AG in our examined intervention group after the 1-year yoga training. The increase in GH in the intervention group after training might be related to the impact of yoga as 'exercise is a powerful stimulus to GH secretion'[369] Lower GH level has been demonstrated to increase abdominal fat, thereby contributing to central obesity. Conversely, overeating has been demonstrated to decrease GH without affecting body weight[389]. Therefore, our results suggest that yoga exercise might favor lipolysis via GH release, as opposed to GH-driven lipogenesis in response to AG stimulation[226]. The metabolic functions of insulin depend partly on other hormones such as GH. In the current study, insulin level remained unchanged in both groups after the experimental period. This might be related to the close age range of the subjects, as age has been proposed to represent a strong predictor of insulin resistance[390]. It is plausible that the pancreatic secretion of insulin in the subjects was maintained irrespective of the level of physical activity or that the functions of insulin were masked by other regulatory molecules including ghrelin gene products and GH as reported in this study. In essence, our study suggests that insulin might not represent a suitable marker for monitoring long-term beneficial effects of yoga in MetS adults with central obesity.

Studies evaluating HOMA-IR in MetS subjects with exercise intervention are limited. In particular, a few studies that described HOMA in MetS subjects with yoga intervention are contradictory[391,392], and this might be related to the short-term duration of the studies, which makes the complex regulation of glucose and insulin homeostasis difficult to interpret. In another study, yoga exercise was asserted to increase the sensitivity of the pancreas islets to glucose signal in subjects who had performed different combinations of yoga postures for 5 consecutive days[393]. In our study, HOMA2-%B, HOMA2-%S, HOMA2-IR and disposition index were not altered in yoga and control groups, although HOMA2-%S tended to increase in yoga group relative to control group. It is noteworthy that fairly constant disposition index which is an indication of normal glucose metabolism was observed in intervention and control groups[394]. Since HOMA model principally describes hepatic insulin resistance, the clamp method which serves as the gold standard for describing insulin resistance might be needed in the future studies to provide clear distinction between peripheral and hepatic insulin resistance[395].

In conclusion, MetS individuals with central obesity who had participated in 1-year yoga exercise training were demonstrated to achieve changes in the proportions of circulatory UnAG, AG, total ghrelin and obestatin. Besides, the effect of yoga training on GH might have suppressed the action of AG on GH, which led to lipolysis and decrease in waist circumference. The present findings support a role of ghrelin gene products and GH in the pathogenesis of MetS and the beneficial cardiometabolic effects of yoga training. It remains to be elucidated whether group gathering or social life might have affected the peptide levels of subjects after 1-year of yoga exercise.

Chapter 6. Protective effect of unacylated ghrelin on compression-induced skeletal muscle injury mediated by SIRT1-signaling

(Remark: The findings presented in this chapter have been Published

Ugwu FN[#], Yu AP[#], Sin TK, Tam BT, Lai, CW, Wong SC and Siu PM. (2017). Protective effect of unacylated ghrelin on compression-induced skeletal muscle injury mediated by SIRT1-signaling. *Frontier's in Physiology*.doi: 10.3389/fphys.2017.00962 #Equal contribution).

6.1. Introduction

Pressure ulcers, also called bedsores or decubitus ulcers, are injuries to soft tissues that resulted from the application of prolonged pressure or friction to the body[396]. These ulcers are often identified and staged by the severity of symptoms, with stage I being the mildest and stage IV being the worst. The prevalence of pressure ulcers among ~6,000 patients surveyed in 5 European countries including Belgium, Italy, Portugal, United Kingdom and Sweden has been reported to be ~18%[397]. The financial burden of pressure ulcers was estimated over £2 billion in the United Kingdom[398]. In the United States, pressure ulcers were shown to be associated with increased mortality, prolonged hospital stays and hospital re-admission[399]. The association of pressure ulcer incidence and increased morbidity and mortality has been substantiated by the observation that approximately 70% of 74 in-patients died within six months of developing pressure ulcers, with an average of 7 weeks from the ulcer onset to death[400]. A recent report indicated that the mortality rate in patients with pressure ulcers was significantly higher than that in patients without pressure ulcers[401]. Furthermore, the staging of pressure ulcer is positively related to the pain severity of patients as well as the cost of treatment[402]. Oot-Giromini et al.[403] noted that the cost of preventing pressure ulcers was significantly less than the cost of treatment of pressure ulcers in long term. Thus, early detection and prevention of pressure ulcers represent cost-effective remedies to alleviate the healthcare problem of pressure ulcers.

Ghrelin is a gastric peptide that exists in two main forms namely acylated ghrelin (AG) and unacylated ghrelin (UnAG). The receptor for AG has been identified as the growth hormone secretagogue receptor (GHSR)[139]. Although UnAG accounts for 80-90% of circulating ghrelin[191], initial reports termed UnAG as an inactive peptide. This is mainly due to the inability of UnAG to activate GHSR at the physiologic level[17] and the lack of identified receptor or mediator that accounts for the activities of UnAG. Until recently, some studies began to reveal the pro-survival role of UnAG but in most instances the receptor or mediator responsible for the reported biological effects of UnAG has not yet been identified.

SIRT1 might be a potential signaling mediator involved in underpinning the cellular effects of UnAG. SIRT1 is a class III histone deacetylase which regulates numerous cellular activities related to various physiological and pathologic signaling pathways[404,405]. In vitro studies conducted by Shimada and colleagues[406] have demonstrated that UnAG prevented microvascular endothelial cell from oxidative stress-induced apoptosis by stimulating SIRT1 signaling pathway. Besides, UnAG treatment has been reported to increase SIRT1 level in endothelial cell of both ischemic and non-ischemic muscles relative in ob/ob obese mice[407]. In vitro studies have also revealed that UnAG protected muscle cell. UnAG has been demonstrated to prevent dexamethasone-induced atrophy in C2C12-derived myotubes via mammalian target of rapamycin complex 2 (mTORC2) pathway[408]. Sheriff and colleagues[409] have also demonstrated that protein catabolism induced by combined treatment of two pro-inflammatory factors, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), in cultured C2C12 myotubes was abolished by UnAG

administration via PI3K signaling pathway. Collectively, there is a dearth of knowledge on the biological effects of UnAG on skeletal muscle. The present study tested the hypothesis that UnAG protects skeletal muscle from pressure-induced injury and the myoprotective effects of UnAG are mediated through SIRT1 signaling pathway.

6.2. Materials and methods

6.2.1. Animals

Sprague Dawley rats (N=15) weighing between 280–300 g were housed in a light, temperature and humidity controlled environment under standard condition, with a 12-hour light-dark cycle and free access to water and chow. Animal husbandry and compression protocol were conducted in full accordance with the approval of the Animal Subjects Ethics Subcommittee of the Hong Kong Polytechnic University.

6.2.2. Compression injury protocol

The induction of pressure ulcer in Sprague Dawley rats was performed in accordance with our established protocol[99]. Briefly, rats were anesthetized with a ketamine and xylaxine mixture via intraperitoneal injection. The hairs on both hind limbs were gently shaved while animals were under anesthesia. Animals were subjected to a 2-day compression protocol. On the first day of compression, a static pressure of 100 mmHg was applied to the tibialis region of the right hind limb for 6-hour via a compression indentor. Anesthesia was ensured throughout the compression by intraperitoneal injection of one-third the initial anesthetic mixture delivered when needed. The left uncompressed hind limb served as intra-animal control. After an 18-hour rest, the entire compression protocol was repeated

on the second day. Animals were sacrificed 18-hour after the second compression via CO_2 ventilated euthanasia. Muscle tissue underneath the compression region and muscle tissue at similar region of the opposite uncompressed limb were harvested and frozen in liquid nitrogen, and stored at -80ºC for further analyses.

6.2.3. Treatment of UnAG and EX527

Animals were randomly assigned into 3 groups (n = 5 per group) and received one of the following treatments for 2 days: saline, UnAG (100 µg/kg, injected before and after compression) and UnAG (100 µg/kg, injected before and after compression) co-treated with EX527 (1 mg/kg injected before compression). EX527 is a potent pharmacological inhibitor of SIRT1. The dose of UnAG adopted in the present study was reported in the previous studies[220,410]. The dose and route of administration of EX527 was adopted from previous study[411].

6.2.4. Histological analysis

Muscle tissues were separated into two halves transversely. Twelve µm-thick frozen muscle tissue cross-sections were prepared in a cryostat at -20°C. Tissue sections were air-dried at room temperature for 30 minutes and fixed with 10% formalin at room temperature for 10 minutes before stained in Mayer's Hematoxylin and 1% Eosin in $CaCl_2$. Thereafter, sections were dehydrated, mounted and examined under a confocal microscope. Interstitial nuclei and muscle nuclei were counted and area of interstitial space was quantified by using ImageJ software (National Institutes of Health, USA). The number of nuclei within myofibers was normalized to the number of myofibers in the field. All the histological data

were presented as average from four random, non-overlapping image fields captured under a 20x objective.

6.2.5. Subcellular protein fractionation and Western blot

Proteins were extracted as previously described[99]. The protein contents were quantified using Bradford Assay. The abundance of interested protein was determined by Western blotting. Briefly, the protein samples were boiled at 95°C in Laemmeli buffer with 5% β-mercaptoethanol, fractioned by SDS-PAGE, transferred onto a PVDF membrane, blocked with 5% milk for an hour and probed with one of following primary antibodies at 4 ºC overnight: anti-SIRT1 (15404, Santa Cruz, Santa Cruz, CA, USA), anti-RIP1 (7881, Santa Cruz), anti-RIP3 (135170, Santa Cruz), anti-NOS2 (650, Santa Cruz), anti-p53 (56179, Santa Cruz), anti-phospho-p53 (101762, Santa Cruz), anti-Bax (493, Santa Cruz) and anti-AIF (13116, Santa Cruz). The membranes were washed three times at an interval of 15 minutes each, probed with the appropriate secondary antibodies, either anti-mouse IgG or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibodies (1:4000; Cell Signaling, Beverly, MA, USA) for 1 hour, followed by three washes and the application of luminal reagent (NEL103001EA, Perkin Elmer, Waltham, MA, USA). The signals were captured via BioRad Chemidoc System. Beta-tubulin was used as the internal control and for normalization.

6.2.6. Apoptotic cell death enzyme-linked immunosorbent (ELISA) assay

The Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN, USA) was employed to determine the apoptotic DNA fragmentation according to the instruction of the manufacturer. In this procedure, the wells of a microplate were coated with primary mouse monoclonal histone antibody in coating solution at 4 °C overnight. Thereafter, samples were incubated in coated wells at room temperature for 90 minutes, followed by washes and incubation with conjugated solution containing secondary peroxidase-conjugated anti-DNA-POD mouse monoclonal antibody. Reaction in the absence of conjugation solution served as the negative control. After washes, 2,2'-azino-di-(3-ethyl-benzthiazoline sulphonate) substrate was added to the microplate to determine the retained amount of peroxidase colorimetrically by measuring the absorbance at a wavelength of 405 nm via a spectrophotometer. The optical density (OD) was normalized to the milligrams of protein used in the assay and presented as apoptotic DNA fragmentation index.

6.2.7. SIRT1 deacetylation assay

Deacetylase activity of SIRT1 was assessed by a fluorometric assay (Cyclex, Nagoya, Aichi, Japan) in accordance to the manufacturer's instructions. Briefly, a reaction mixture containing 1 mM fluoro-substrate peptide, 5 mAU lysylendopeptidase, 2 mM NAD^+, 50 mM Tris-HCl, 0.5 mM DTT-containing SIRT1 assay buffer, 1 μm Trichostatin A (a NAD^+-independent histone deacetylase inhibitor) was prepared. The reaction was initiated by adding 5 μl protease inhibitor-free muscle protein extracts under thorough mixing. Fluorescence intensity (excitation 340 nm, emission 460 nm) was measured by a

microplate fluorometer (Infinite F200, Tecan, Mannedorf, Switzerland). All readings were normalized to 1 mg of protein content of the respective samples.

6.2.8. Statistical analyses

Statistical analyses were performed using the SPSS 22.0 software package (IBM, Chicago, IL, USA). In all experimental studies, the contralateral limb served as the intra-animal control. A normality test was performed to examine data distribution. Normally distributed data were compared using one-way analysis of variance [411] followed by Tukey's Honestly Significant Difference (HSD) post hoc test. Non-normal data were analyzed by Kruskal-Wallis H test followed by Dunn-Bonferroni post hoc test. All data were expressed as mean $\pm$ standard error of the mean. Significance level was set at $p < 0.05$.

6.3. Results

6.3.1. Unacylated ghrelin attenuated histopathological alterations of skeletal muscle after compression

The effects of compression injury and drug treatment on muscle histology are shown in the representative pictures (Figure 6.1A). The area of interstitial space in compressed muscle was increased by 2.7 folds when compared to uncompressed muscle in saline group (Figure 6.1B). UnAG, but not in combination with EX527, prevented the elevation of the area of interstitial space in the compressed muscle (Figure 6.1B). Our histological results revealed that the number of interstitial nuclei was 18.9 folds higher in the compressed muscle relative to the uncompressed muscle in saline group (Figure 6.1C). The increase in the number of interstitial nuclei was attenuated by UnAG (Figure 6.1C). The number of muscle

nuclei was not significantly different between compressed and uncompressed muscles in all groups (Figure 6.1D). The total number of nuclei was significantly different between compressed and uncompressed muscles in all group except the group treated only with UnAG. The total number of nuclei in compressed muscle was increased by 1.9 fold when compared to the uncompressed muscle in saline group (Figure 6.1E). Furthermore, the number of muscle nuclei normalized to the number of myofibers was not changed after compression in all groups (Figure 6.1F).

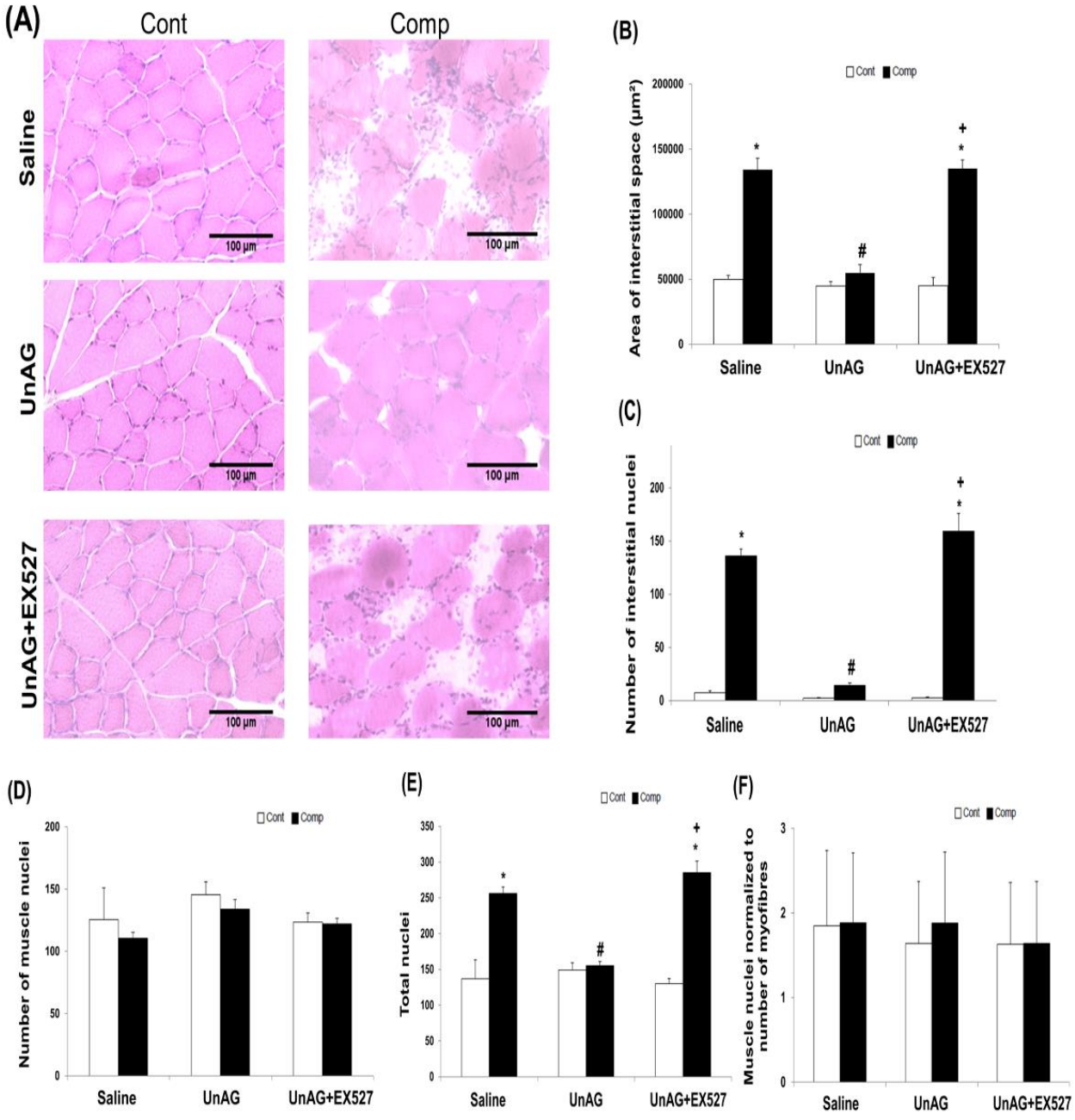

Figure 6. 1 Histological analyses

UnAG abolished abnormal muscle histology induced by moderate compression but this effect was vanished when co-treated with EX527 (A). The elevation of area of interstitial space induced by compression injury was alleviated by UnAG (B). The reduction of the number of interstitial nuclei induced by UnAG in the compressed muscle was mitigated by the co-treatment of EX527 (C). No compression or treatment effect was observed for the number of muscle nuclei (D). The total number of nuclei was significantly different between compressed and uncompressed muscles in all groups except in the group treated only with UnAG (E). Neither compression nor drug treatment affected the number of muscle nuclei normalized to the number of myofibers (F). *$p<0.05$, compressed muscle compared to uncompressed control muscle; #$p<0.05$, UnAG compared to Saline; +$p<0.05$, UnAG+EX527 compared to UnAG; Cont, control muscle; Comp, compressed muscle.

6.3.2. Unacylated ghrelin abolished the increase in necroptosis proteins exerted anti-apoptotic effects and preserved SIRT1 enzymatic activity in compressed muscle

To investigate the necroptosis signaling, RIP1 and RIP3 protein abundances were examined. In muscle of saline group, RIP1 was significantly increased by 1.8 folds in response to compression (Figure 6.2D). This compression-induced elevation of RIP1 was abolished by UnAG treatment but not in conjunction with EX527 (Figure 6.2D). Similarly, RIP3 was significantly increased by 2.5 folds in the compressed muscle relative to uncompressed muscle in the saline group (Figure 6.2E). The elevation of RIP3 was attenuated by UnAG treatment but not in combination with EX527 (Figure 6.2E). In saline group, the protein abundances of total p53, phospho-p53, Bax, AIF, and phosphorylation ratio of p53 were increased in the compressed muscle by 1.9 folds, 18.7 folds, 3.9 folds, 4.8 folds, and 9.8 folds, respectively, relative to the uncompressed muscle (Figure 6.2F – I). These increases were all attenuated by UnAG treatment but not in combination with EX527 (Figure 6.2F – I). Similarly, apoptotic DNA fragmentation index was found to be 7.4 folds elevated in the compressed muscle when compared to the uncompressed muscle in saline group (Figure 6.2K). UnAG treatment, but not in combination with EX527, was observed to inhibit the increase in apoptotic DNA fragmentation index as induced by compression (Figure 6.2K). The protein content of NOS2 was significantly elevated in the compressed muscles relative to the uncompressed muscles in all groups (Figure 6.2L). The protein content of NOS2 was significantly elevated by 3.8 folds, 3.6 folds, and 6.1 folds in the compressed muscles relative to the uncompressed control muscles in saline, UnAG,

and UnAG+EX527 groups, respectively, (Figure 6.2L). SIRT1 protein abundance was tended to decrease in compressed muscles when compared to the uncompressed muscles in saline group ($p=0.09$) (Figure 6.2M). The protein abundance of SIRT1 was significantly decreased in the compressed muscle relative to the uncompressed muscle in UnAG+EX527 group. In saline group, the deacetylase activity of SIRT1 was significantly decreased by 72% in the compressed muscle relative to the uncompressed muscle (Figure 6.2N). This compression-induced decrease in SIRT1 deacetylase activity was not found in the compressed muscle treated with UnAG (Figure 6.2N).

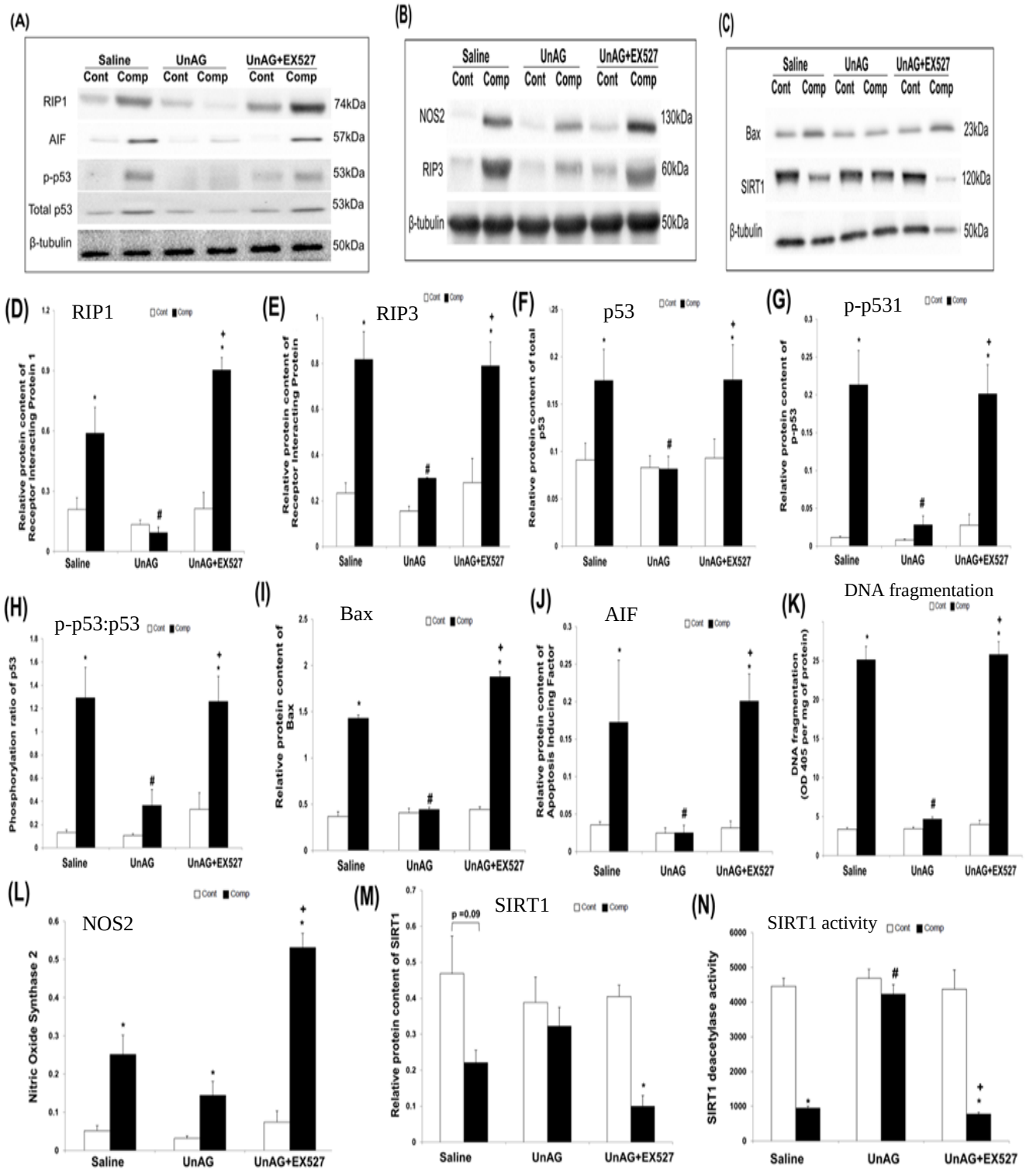

Figure 6. 2 Immunoblot and biochemical analyses

For the immunoblotting, all samples were probed for the examined protein with the respective antibody on the same membrane which underwent the same probing and washing procedure. The representative blot pictures are shown in A, B and C. UnAG, but not in conjunction with EX527, blunted the increase in protein abundance of RIP1 in compressed muscle (D). Similar pattern was observed in the protein abundance of RIP3 (E). The immunoblot analyses revealed the suppressive effects of UnAG, but not in conjunction with EX527, on the abundances of p53 (F), phospho-p53 (G), phosphorylation ratio of p53 (H), Bax (I) and AIF (J). The anti-apoptotic effect of UnAG was confirmed by apoptotic DNA fragmentation index (K). The protein content of NOS2 was significantly elevated in the compressed muscles in all groups, irrespective of drug administered (L). SIRT1 protein abundance was tended to decrease in the compressed muscle when compared to uncompressed muscle in saline group (M). SIRT1 protein abundance was significantly decreased in muscle co-treated with UnAG and EX527 after compression (M). The deacetylase activity of SIRT1 was significantly decreased after compression in all groups except in compressed muscle treated only with UnAG (N). *$p<0.05$, compressed muscle compared to uncompressed control muscle; #$p<0.05$, UnAG compared to Saline; +$p<0.05$, UnAG+EX527 compared to UnAG; Cont, control muscle; Comp, compressed muscle.

6.4. Discussion

The present study demonstrated the protective effect of UnAG on muscle compression injury as shown by the maintenance of muscle histology after prolonged moderate compression. Our results illustrated that the protective effect of UnAG on muscle compression was attributed to the favorable alterations of necroptosis and apoptosis signaling in skeletal muscle, which probably involved the mediation of SIRT1 signaling pathway.

Studies from our laboratory have demonstrated that UnAG prevented doxorubicin-induced myocardial apoptosis and fibrosis[200] exerted protective effect on diabetic cardiomyopathy[412] and inhibited doxorubicin-induced apoptosis in skeletal muscle[16]. A recent investigation conducted by Cappellari and colleagues revealed that a 4-day administration of UnAG in lean rats inhibited the production of inflammatory cytokines and mitochondrial reactive oxygen species in gastrocnemius muscle[291]. Notably, the specific molecule or receptor that mediates the reported protective effects of UnAG has not yet been recognized. SIRT1 has been demonstrated to regulate pro-survival events and play roles in lifespan extension, apoptosis, age-related disorders, inflammation, and cancers[404,405]. Here, we reported a possible role of SIRT1 in mediating the myoprotective effects of UnAG on compression-induced muscle injury.

Necrosis is a complex form of caspase-independent cell death whereas necroptosis is the term used to describe necrosis that is dependent on necroptosis proteins RIP1 and RIP3[413]. Different factors including oxidative stress, chemical toxin and DNA damage are known

to induce necrotic cell death[413] and the examination of RIP1 and RIP3 serves as a marker for determining necroptosis[414,415]. In this study, the observed activation of necroptosis might be related to ischemia-reperfusion injury in accordance with the findings reported by Fiorillo and colleagues[416] that hypoxia-reoxygenation induced necrosis-dependent activation of poly(ADP-ribose) polymerase 1 (PARP1). According to our observation that UnAG downregulated both necroptosis proteins in the absence of the inhibitor for SIRT1, we interpreted that UnAG might have maintained the cyto-architecture of the compressed muscle by attenuating necroptosis, which is a cellular destructive process. Our data indicated that the interaction of UnAG with SIRT1 might play a role in coordinating necroptosis during pressure-induced tissue injury, suggesting that SIRT1 might be able to serve as the potential molecular target for further unravelling the pathogenesis of pressure ulcers as well as the myoprotective effect of UnAG in pressure ulcers.

Reactive oxygen species and antioxidants are opposing forces that are appropriately balanced under healthy conditions[417]. NOS2 is an enzyme that promotes the formation of large quantities of nitric oxide, a free radical which has a high affinity for other free radicals[418]. For instance, the interaction of nitric oxide with superoxide produces peroxynitrite, which causes protein nitration and DNA damage[419]. Studies a decade ago indicated that the peripheral administration of AG attenuated NOS2 protein expression which was upregulated by gastric ischemic injury in rats, and decreased in vivo reactive oxygen species generated by human polymorphoneuclear cells[420]. In addition, AG has been demonstrated to enhance the expression of key antioxidant enzymes including zinc and manganese superoxide dismutases, catalase, glutathione peroxidase, and glutathione

reductase but reduce expression of NOS2 in liver[421]. Although the beneficial effects of AG on the antioxidant enzymes as well as NOS2 were examined in the absence of any pathophysiological condition, previous findings suggested that AG might have regulated the oxidative stress enzymes and NOS2 via Akt/ERK1/2 pathway. It remains to be elucidated whether administration of AG or UnAG could confer antioxidative protection in skeletal muscle subjected to compression-induced injury. Studies from our laboratory revealed that the transcript content of NOS2 was elevated in skeletal muscle after compression[99]. Recently, UnAG was found to preserve gastrocnemius muscle mass in male Wistar rats with chronic kidney disease (as induced via nephrectomy) by normalizing the mitochondrial reactive oxygen species production, inflammatory mediators and insulin signaling[291]. Nonetheless, our results showed that UnAG treatment did not reverse the increased abundance of NOS2 in the compression-injured skeletal muscle. Instead, our findings suggested a role of SIRT1 in mediating the effect of UnAG on NOS2 expression as simultaneously, UnAG tended to decrease NOS2 but increased SIRT1 activity in the absence of EX527. In vitro study has revealed that SIRT1 deletion elevated the expressions of two hypoxia-responsive genes, interleukin 1β and NOS2[422]. Hypoxia is a fundamental pathology for ischemic-reperfusion diseases including pressure ulcers. Given that UnAG tended to reduce NOS2 expression, possibly by interacting with SIRT1, our data suggest that at higher doses, UnAG may exert a beneficial effect by diminishing hypoxia-related pathologies associated with compression-induced muscle injury.

Previously, we reported the activation of apoptosis in conjunction with pathohistological damage in skeletal muscle in response to moderate compression[423]. Additionally, we demonstrated that UnAG blunted the apoptotic alterations including elevations of apoptotic DNA fragmentation index and number of TUNEL-positive nuclei and the upregulation of Bax in the muscle exposed to doxorubicin[16]. The tumor suppressor protein p53 is known to be involved in DNA damage signaling and is a SIRT1 molecular target[424]. The notion that p53 contributes to the modulation of apoptotic signaling in ischemia-reperfusion injury[425] further stimulated our interest in examining its signaling events in the present muscle compression injury study. As a molecular sensor of cellular stress, p53 is activated and phosphorylated on serine-15[426] and then executes Bax-associated apoptotic signal transduction[427]. In this study, we demonstrated that the contents of total p53, phospho-p53 and p53 phosphorylation ratio were significantly elevated in the compressed muscle in saline control and SIRT1-inhibitor groups, but not in muscle treated only with UnAG. The increase in apoptotic DNA fragmentation index and simultaneous elevation of pro-apoptotic protein Bax in response to p53 phosphorylation is consistent with previous reports[99,423,428]. Our finding that p53 expression was blunted in muscle treated with UnAG but was significantly elevated in the compressed muscle relative to the uncompressed muscle in saline and SIRT1-inhibitor groups, suggesting that SIRT1 was probably involved in mediating the protective effect of UnAG. These results are consistent with the findings of Pinton and co-workers[429] demonstrated that UnAG-induced SIRT1 expression resulted in p53 deacetylation and protection of endothelial cell against senescence in ischemic condition. The decrease in SIRT1 deacetylase activity in the saline and SIRT1-inhibitor-

treated muscles after moderate compression might have resulted from the complicate process of ischemia-reperfusion injury including reactive oxygen species, calcium overload, endothelial dysfunction, inflammatory response[430], and regulation via microRNAs[431]. These findings indicated that the possible deacetylation of p53 by SIRT1, in response to UnAG, might provide clues for future investigation of the underlying mechanisms of pressure-induced tissue injury and pressure ulcers. Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein which, upon release, is capable of causing chromatin condensation and DNA fragmentation in nucleus[432]. Modjtahedi and colleagues[433] observed that the contribution of AIF to cell death induction depended probably on cell type and death triggers. We reported here that, in response to compression injury, AIF was concomitantly elevated with the increased Bax expression except in muscle treated with UnAG. Multi-domain pro-apoptotic proteins Bax and Bak play essential roles in mitochondrial outer membrane permeability, leading to release of AIF in response to cell death trigger[434]. Also, in response to oxidative stress and DNA damage, nuclear poly(ADP-ribose)polymerase-1(PARP-1) is activated, leading to the release of AIF from the mitochondria[435,436]. In this study, we speculate that UnAG might have partly blunted AIF by inhibiting the upstream regulators of apoptosis including p53 and Bax as well as stimulating SIRT1 enzymatic activity. Further study is warranted to examine this speculation and to reveal the exact interaction of UnAG with the p53-Bax-AIF apoptotic signaling axis.

In conclusion, our data demonstrated that UnAG preserved muscle histology and blunted the alterations of necroptosis and apoptosis signaling in response to compression injury. The observed protective effects were probably associated with the SIRT1 signaling based on the observation that the effects of UnAG were mostly abolished in the presence of the potent pharmacological inhibitor of SIRT1. Acute cell loss might result from excessive cell death due to ischemia-reperfusion injury[437], which is a hallmark of pressure-induced tissue injury and pressure ulcers. The present study provided novel data contribute to the understanding of necroptosis and apoptosis on the myoprotective effect of UnAG on pressure ulcers. As UnAG preserved muscle architecture by blunting the compression-induced necroptosis and apoptosis, UnAG might be worth to be further explored as one of the targets to develop new therapeutic regimens.

Chapter 7 Overall discussion, limitations and future perspectives

7.1. Overall discussion

This thesis aims to investigate the role of ghrelin in metabolic and muscle disorders. To achieve the aims, circulatory GH, IGF-1, nesfatin-1 and ghrelin gene products including UnAG, AG and obestatin were evaluated in adults with central obesity, hypertension and MetS. In addition, UnAG was specifically investigated as a therapeutic intervention in pressure ulcer disease. Followings are the major findings of different studies. Obestatin, obestatin/ghrelin ratios and GH reveal the interaction of central obesity and other cardiometabolic risk factors of MetS (Chapter 3); obestatin demonstrates the interaction effect of hypertension with central obesity, with central obese-only subjects reporting higher obestatin level compared to non-central obese normotensive subjects and central obese hypertensive subjects (chapter 4); 1-year yoga training alters circulatory ghrelin gene products and GH in adults with MetS (chapter 5); finally, UnAG prevents pressure-induced injury in skeletal muscle via SIRT1-signaling pathway (Chapter 6). Metabolic and muscle disorders, as well as ghrelin axis were reviewed comprehensively in chapter 2. Besides the above comprehensive summary, this section discusses the overall findings and inter-relationship between studies.

Preliminary studies identified AG as the most important product of ghrelin axis, especially as the physiological properties of UnAG could not be appropriately described. Several independent studies have implicated AG in the regulation of food and body weight. For instance, subcutaneous daily administration of AG induced adiposity by reducing lipolysis,

whereas intracerebroventricular administration of AG resulted in a dose-dependent increase in food intake and body weight in mice and rats[226].

However, the past decade has witnessed renewed interest in the physiological role of UnAG. UnAG has been shown to preserve and promote pancreatic β-cell function[154]. UAG prevented β-cell destruction and restored glucose homeostasis in streptozotocin-treated newborn rats compared to control rats[351]. These findings suggested that UnAG might be important in the management of glucose-related disorders. Recently, our laboratory demonstrated that UAG restored impaired insulin signalling and normalized suppressed autophagic signalling in skeletal muscle of diabetic mice in conjunction with decreased fasting glucose and body weight[438]. Based on promising results from several animal studies, synthetic UnAG analogs have been recently developed for the treatment of metabolic disorders. AZP-531, a cyclic UnAG analog, has been investigated for the treatment of cardiometabolic disorders. Two-week administration of AZP-531 was shown to improve glucose concentration without increasing insulin level, indicating an insulin-sensitizing effect of AZP-531 in overweight/obese subjects[387]. In addition, AZP-531 was demonstrated to decrease mean body weight in healthy and overweight/obese subjects, as well as subjects with T2DM[387]. Although existing literature have not demonstrated UnAG-driven GH release, this thesis has revealed similar profiles of UnAG and growth hormone in subjects with central obesity, hypertension or MetS and normotensive non-obese subjects. Intriguingly, intracerebroventricular administration of UnAG has been demonstrated to stimulate feeding during the light and dark phases in rats[184]. In the same study, intracerebroventricular administration of UnAG stimulated feeding in GHSR-deficient mice

and their wild-type littermates compared to wild-type control mice[184]. Hence, it appears that UnAG might potentiate GH and both peptides might be simultaneously altered in different types of metabolic disorders including hypertension, central obesity and MetS.

A few hypothalamic sites including the arcuate and paraventricular hypothalamic nuclei play important roles in the modulation of energy balance[439]. Although stomach is the principal site for the production of ghrelin, electron microscopic findings suggested that ghrelin might also be secreted by the hypothalamus. The axon terminals of several hypothalamic regions, including the arcuate and paraventricular hypothalamic nuclei have been shown to contain ghrelin[440]. Also, ghrelin has been shown to stimulate the release of neuropeptide Y from hypothalamic axon terminals, leading to increase in food intake and decrease in energy expenditure[440]. These findings provided anatomic evidence for interactions between ghrelin and the hypothalamus in the regulation of energy balance. It has been shown that AG infusion in healthy volunteers decreased mean arterial pressure, increased circulating adrenaline, adrenocorticotropin and cortisol but had no effect on heart rate or noradrenaline compared to placebo-infused control subjects volunteers[220]. In a separate investigation, intravenous infusion of AG decreased mean arterial pressure, tended to increase circulating adrenaline, adrenocorticotropin and cortisol but failed to alter heart rate and noradrenaline plasma levels in subjects with chronic heart failure compared to placebo-infused control subjects failure[441]. In these studies, the authors hypothesized that AG might directly induce vasodilation in blood vessels and prevent the activation of the sympathetic nervous system during hypotension. The observed decrease in mean arterial blood pressure prompted investigation into AG agonists. In a clinical trial, intravenous

infusion of TZP-101, an AG agonist, increased plasma adrenaline without affecting noradrenaline, but decreased mean arterial blood pressure and heart rate from baseline approximately 45 to 60 minutes after infusion[442]. The decrease in heart rate and mean arterial blood pressure in the clinical trial was attributed to upregulation of the parasympathetic nervous system, as opposed to vasodilation and inhibition of the sympathetic nervous system previously speculated for AG[220,441]. In our second study, the decreased AG in subjects with hypertension and central obesity compared to subjects with neither hypertension nor central obesity or subjects with only obesity might be partly related to nitric oxide which is an important regulator of energy balance. The role of nitric oxide in ghrelin-mediated increase in appetite is buttressed by a study showing that central AG infusion increased hypothalamic concentration of nitric oxide synthase[443]. Similarly, systemic bioavailability of nitric oxide was shown to be decreased in obese subjects with hypertension when compared with normal weight controls[444]. Other studies have also demonstrated decreased in vivo production of nitric oxide in obese subjects with metabolic syndrome when compared with normal-weight subjects and obese subjects without metabolic syndrome[445,446]. Compelling evidence has also revealed that nitric oxide estimates including plasma nitrate and nitrite levels were decreased in obese mice with hypertension in comparison to obese normotensive mice[447]. Our findings suggested that reduced AG might have been influenced by obesity-related hypertension and from the foregoing discussion, the decrease in AG in subjects with hypertension irrespective of central obesity might be partly related to a reduced nitric oxide bioavailability. Addition investigation is warranted to explore this intriguing research topic.

The controversy surrounding previous reports of the physiological effects of obestatin suggests that obestatin might be properly interpreted in the light of UnAG and AG. Consistent with this notion, the current thesis investigated ghrelin/obestatin ratios in subjects with no MetS risk factor, no MetS risk factor except central obesity, all 5 MetS risk factors except central obesity and all 5 MetS risk factors including central obesity. We found higher obestatin level was increased, whereas UnAG/obestatin, AG/obestatin and total ghrelin/obestatin ratios were decreased in subjects with the cluster of all MetS risk factors compared to subjects with no MetS risk factor. The current thesis also evaluated ghrelin/obestsin ratios in obese, hypertensive, hypertensive-obese and normotensive non-obese subjects. We found that obestatin level was increased, whereas UnAG/obestatin, AG/obestatin and total ghrelin/obestatin ratios were decreased in subjects with hypertension and central obesity compared to normotensive non-obese subjects. Thus results from this thesis demonstrates that obestatin increase might parallel the number of MetS risk factors. Despite being a controversial peptide, results from this thesis suggest that the multifunctional actions of obestatin might be better interpreted in relation to AG, UnAG and total ghrelin.

Although balanced diet and exercise are recommended for the management of several metabolic disorders[356], exercise represents the preferred means to delay the manifestation of MetS risk factors[448]. Although numerous studies linking each MetS risk factor to exercise have been conducted, data focusing on the association between MetS and exercise are relatively sparse in the literature. One epidemiologic study that evaluated the association between MetS and exercise was the ATTICA Study[449]. The results of ATTICA Study

revealed that light or moderate leisure time physical activity was associated with a reduction in the prevalence of MetS in over participants. Interestingly, data from the ATTICA study showed that regular, intensive exercise was associated with a much greater decrease in the prevalence of MetS[449]. Results from this thesis also suggest that long-term exercise such as yoga exercise might benefit the body via homeostatic control of ghrelin gene products. Our previously published reports that yoga exercise decreased waist circumference and findings from this thesis that yoga intervention stabilized UnAG parallel evidence that UAG and its synthetic analogs decreased weight without affecting food intake[193,438]. Ultimately, the therapeutic potential of UnAG in metabolic disorders is likely to receive greater attention within the next few years. The current thesis supports proposals for production of synthetic analogs capable of increasing production of UnAG from ghrelin gene, for the management of several metabolic disorders.

Research directly linking MetS risk factors and pressure ulcers are lacking. However, existing literature suggests that MetS risk factors might aggravate the conditions of individuals who suffer from pressure ulcers. For instance, pressure ulcer might be worsened in older individuals with MetS when compared with older subjects without MetS, as increasing age is a common denominator of both MetS and pressure ulcer. Also, closer inspection reveals similar molecular mechanisms of pathology for MetS and pressure ulcer. For instance, several MetS risk factors including central obesity, hypertension, dyslipidaemia, hyperglycemia and insulin resistance have been linked to formation of plaques in the vessels[450], whereas mechanical pressure in pressure ulcer is known to partially occlude blood vessels[97]. Interestingly, vascular plaques due to MetS complications and

vascular occlusion due to pressure ulcer promote ineffective tissue perfusion and abnormal cellular exchange of nutrients and wastes[451], leading to cell death. A recent study demonstrated that administration of UnAG to nephrectomized uremic rats and myotubes treated with human uremic serum normalized mitochondrial ROS generation, restored insulin signaling and upregulated autophagy marker, LC3II/I ratio[452]. Hence, UnAG might positively modulate outcome in pressure ulcer by preventing oxidative stress as evidenced by this thesis and prevent obesity-associated skeletal muscle oxidative stress and insulin resistance by upregulation of autophagy[452]. UnAG might therefore represent a novel potential treatment for pressure ulcer and metabolic disorders including central obesity.

7.2. Limitations and recommendations

There are several limitations in the present studies. The absence of menstrual cycle data of female subjects with active reproductive cycle is one limitation of the current thesis. In our study of the interaction of central obesity with the other MetS risk factors, the number of subjects with all 5 MetS risk factors except central obesity was 10 (Chapter 3). This relatively small number of participants was a limitation of the study. A higher number of participants would increase the power of the study. We examined the interaction of hypertension and central obesity in only female subjects (Chapter 4). Sex differences in hypertension among young adults have been reported[453]. Among 14,497 participants, hypertension was recorded in approximately 27% and 12% of men and women respectively[453]. Similarly, the influence of obesity on cardiac morphology has been shown

to be greater in females compared to males, mainly in the presence of arterial hypertension[344]. Therefore, gender consideration in future studies is recommended for precise interpretation of obesity-related hypertension studies. This thesis defined obesity based on waist circumference alone. Future studies might incorporate other estimates of central obesity including BMI, waist-hip ratio, waist-height ratio and sagittal abdominal diameter. Further studies are recommended to gain insights into the formation of AG via octanoylation by GOAT and the deoctanoylation of AG to UnAG in the tissues.

Pressure ulcer is a complicated disease with several highly regulated processes. Although the receptors and biochemical pathways of UnAG remain elusive, this thesis reveals that UnAG protects muscle from compression induced injury by SIRT1 signaling pathway (Chapter 6). It is widely acknowledged that inflammation is extensively connected to different cell death and survival pathways. Our lab previously demonstrated the opposing responses of apoptosis and autophagy to moderate compression in skeletal muscle of rats[454], suggesting that cell death and survival pathways might be simultaneously activated in pressure ulcer. More importantly, a recent study reported that ATG5 gene silencing of myotubes treated with human uremic serum blocked the protective effects of UnAG against mitochondrial ROS generation and impaired insulin signalling[452]. Further studies are warranted to precisely define the molecular mechanisms involved in UnAG-mediated effects on pressure induced deep tissue injury, and to investigate the potential use of UnAG to treat subjects at risk or suffering from pressure ulcer. Also, future research is recommended to explore the interconnections of necrosis, apoptosis, autophagy and inflammation in pressure ulcer and the role of UnAG in the complex events.

There is still global difficulty in explaining the complete mechanisms that underlie various diseases including pressure ulcer, hypertension, obesity, cancer and diabetes in humans. This is partly due to the difficulty encountered in extrapolating data from studies on several animal models which differ substantially from human metabolism, even though humans share very similar genetic properties with these animals. Given the crucial insight regarding ghrelin and obestatin roles in metabolic disorders, as evidenced in this thesis, we recommend further investigations using ghrelin gene, GHSR and GOAT-conditional knockout mouse models, in relation to central obesity, hypertension and MetS. It is probable that careful regulation of ghrelin gene products and GH might normalize skeletal muscle homeostasis, thereby preventing manifestation of several metabolic disorders.

7.3. Conclusions

The present thesis has investigated the role of ghrelin axis in cardiometabolic disorders and pressure-induced deep-tissue injury. The findings in this thesis suggest that cardiometabolic disorders might be attributed to the complex relationship between central obesity and the cluster of the other 4 MetS risk factors or between hypertension and central obesity in susceptible individuals; determining how ghrelin gene variants and GH are modulated by hypertension and central obesity might further unravel the roles of these peptides in blood pressure and body weight regulations; long-term yoga exercise might modulate circulatory ghrelin gene products even in the absence of favourable glycemic and insulin responses; and UnAG might effectively protect skeletal muscle from pressure-induced deep tissue

injury via SIRT-1 signaling pathway. The prevalence of metabolic disorders is increasing alarmingly in different parts of the globe. Metabolic diseases affect overall quality of life and the costs of managing these diseases are high. With the rise in prevalence of metabolic disorders, the understanding of metabolic pathways is required to develop new therapies for the management of metabolic disorders. Results from this thesis indicate that ghrelin axis is linked to central obesity, hypertension and MetS. As the current thesis reveals that ghrelin gene products and GH are altered in various metabolic disorders, the measurement of these peptides might serve as surrogate indicators to assess the state of health of individuals in future metabolic disorder studies. Collectively, these significant findings support development of novel therapeutics of ghrelin axis for the prevention or treatment of metabolic and muscle disorders.

Chapter 8. List of references

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