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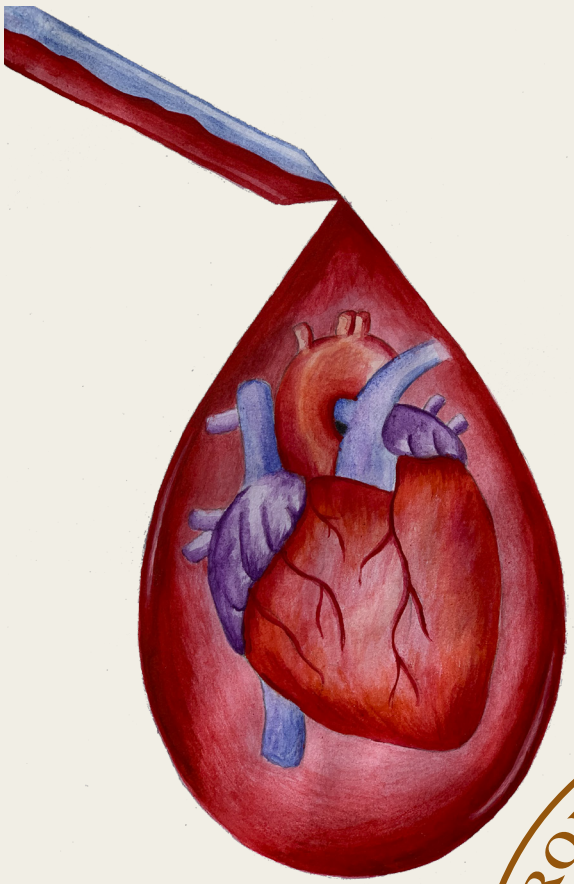

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Ayesha Fawad

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

The burden of cardiometabolic diseases is increasing worldwide. Early detection of high-risk individuals is essential for appropriate diagnosis, better treatment outcomes, quality of life and lower health related costs.

Aims: The overall aim of this thesis is to investigate the possible associations between plasma proneurotensin (Pro-NT) and risk of cardiovascular disease (CVD), type 2 diabetes (T2D), impaired glucose regulation, as well as diet-induced obesity in different study populations. As Pro-NT appears to be a novel risk marker for cardiometabolic morbidity and mortality, we also wanted to study the effects of dietary fat intake on circulating plasma Pro-NT and triglyceride levels. This thesis is based on epidemiological data from three population-based cohorts, The Malmö Preventive Project (MPP), The Malmö Diet and Cancer study- Cardiovascular Cohort (MDC-CC) and The MEDIM cohort (impact of Migration and Ethnicity on Diabetes in Malmö).

Paper I, in this cohort including an elderly population, we identified that Pro-NT predicts incident CVD in both genders, but incident T2D in women only, after 5.4 years of follow up.

Paper II, in a population based cohort of Iraqi and Swedish born men and women, higher Pro-NT levels were observed both in the Iraqi- vs Swedish-born group. However, elevated plasma Pro-NT was associated with impaired glucose regulations assessed as insulin secretion and action and HbA1c in the Iraqi-population only.

Paper III, we found prompt increases in plasma Pro-NT every hour for four hours, after an oral lipid load in healthy individuals. Post-lipid rise of circulating plasma Pro-NT correlated with the changes in plasma triglyceride levels, irrespective of cream and olive oil.

Paper IV, in this longitudinal study, we identified that high Pro-NT levels predicted all-cause mortality (ACM) and cause- specific mortalities (CSM) due to CVD, gastrointestinaltract (GIT) diseases, mental and behavioral diseases, and diseases of unspecific causes, in both gender.

Conclusion:

This thesis shows that circulating Pro-NT is a biomarker that in an elderly population predicts CVD in both gender, but T2D in women only . Irrespective of gender, Pro-NT predicts ACM and CSM. Pro-NT is more strongly associated with impaired glucose regulation in a Middle Eastern immigrant population and may partly explain the increased T2D in this group. Furthermore, oral lipid intake increases Pro-NT levels, which facilitates triglyceride increase in blood in healthy individuals, supporting intestinal lipid absorption as being one key action of Pro-NT.

Key words: Cardiovascular diseases, cohort study, diabetes, insulin resistance, mortality, obesity, proneurotensin (Pro-NT), triglycerides, Middle East, oral lipid load.

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Cover photo by the talented artist Dr. Suneela Zaigham. Cover concept by Ayesha Fawad. The cover photo depicts a blood drop from a pipette representing a simple blood test and a happy healthy heart in that blood drop.

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اهْدِنَا الصِّرَاطَ الْمُسْتَقِيمَ

“Guide us to the straight path”

Al- Quran 1:6

Dedicated to my parents (May their souls rest in peace) and my family.

Content

List of Papers

The papers included in this thesis are listed below. They are reproduced with the permission from the publishers for the thesis.

Paper I: Fawad A, Bergmann, A, Struck J, Nilsson PM, Orho-Melander M, Melander O. Proneurotensin predicts cardiovascular disease in an elderly population. *J. Clin. Endocrinol. Metabol.* 2018**;** 103(5):1940-1947

Paper II: Fawad A, Nilsson PM, Struck J, Bergmann A, Melander O, Bennet L. The association between plasma proneurotensin and glucose regulation is modified by country of birth. *Sci Rep* 2019; 9(1): 13640.

Paper III: Fawad A, Fernandez C, Bergmann A, Struck J, Nilsson PM, Bennet L, Orho-Melander M, Melander O. Magnitude of rise in proneurotensin is related to amount of triglyceride appearance in blood after standardized oral intake of both saturated and unsaturated fat. *Lipids Health Dis.* 2020;19(1): 191.

Paper IV: Fawad A, Bergmann A, Schulte J, Butt ZA, Nilsson PM, Bennet L, Orho-Melander M, Melander O. Plasma proneurotensin and prediction of cause-specific mortality in a middle-aged cohort During long-term follow-up. *J Clin Endocrinol Metab*. 2022;107(3): e1204–11.

List of Abbreviations

ACM	All-cause mortality
AHT	Antihypertensive treatment
BMI	Body mass index
CI	Confidence interval
CIR	Corrected insulin response
CVD	Cardiovascular disease
CHD	Coronary heart disease
CNS	Central nervous system
CSM	Cause-specific mortality
DBP	Diastolic blood pressure
DIo	Oral disposition index.
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter 4
HbA1c	Glycosylated Haemoglobin A1C
HDL-C	High-density lipoprotein cholesterol
HOMA	Homeostatic model assessment
HR	Hazard ratio
ICD	International Classification of Diseases
ISI	Insulin sensitivity index
LDL-C	Low-density lipoprotein cholesterol
MEDIM	The impact of Migration and Ethnicity on Diabetes in Malmö
MDC	Malmö Diet and Cancer study
MDC-CC	Malmö Diet and Cancer study–Cardiovascular Cohort
MHR	Malmö HbA_{1c} register
MPP	Malmö Preventive Project
MR	Mendelian randomization
MI	Myocardial infarction

MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NDR	Swedish National Diabetes Register
NT	Neurotensin
NTR	Neurotensin receptor
OGTT	Oral glucose tolerance test
OR	Odds ratio
Pro-NT	Proneurotensin
PUFA	Polyunsaturated fatty acids
RR	Relative risk
SFA	Saturated fatty acids
SBP	Systolic blood pressure
SD	Standard Deviation
Sd-LDL	Small dense lipoprotein
SGLT2	Sodium-glucose cotransporter-2
T2D	Type 2 Diabetes
TG	Triglycerides
TRL	Triglyceride rich lipoprotein
VLDL	Very low-density lipoprotein cholesterol
WHO	World Health Organization

1 Introduction

Is it bad to be overweight? Is obesity a medical disorder? Historically, the answers to these questions were already explored as early as 460 BC when the Greek physician Hippocrates wrote, *"Corpulence is not only a disease itself, but the harbinger of others"* and *"those who are constitutionally very fat are more apt to die quickly than those who are thin"* (Hippocratic Corpus). Despite this fact, in certain societies, being obese was one of the privileges of the upper classes. In the 18th century, John Hunter collected anecdotes and specimens of human abnormalities including the portrait of the obese Daniel Lambert, found in the Hunterian Museum, Royal college of Surgeons in London.

Figure 1: Portrait of Daniel Lambert by Benjamin Marshall (1768-1835), photo credit: Leicester Museums and Galleries, licensed under CC BY-NC-ND.

Epidemiological studies are used to evaluate the specific disease determinants and its distribution in the specified population. Population-based epidemiological studies play a vital role in determining the biomarkers of various diseases. Thanks to many large population-based studies over the last decades, we have gained a better understanding of the morbidity and mortality risks associated with cardiometabolic diseases and associated conditions including cardiovascular diseases, diabetes, and dyslipidaemia. Much research focus has been given to both primary and secondary prevention of cardiometabolic diseases in individuals with a known increased risk of these conditions.(1) Even though it is important, it is a challenge to identify individuals without any clinical signs of the disease or illness, who run an increased risk of developing the disease.

Study of fasting Proneurotensin (Pro-NT) and its potential harmful effects on human health has opened a new pathway for further research and studies. In this thesis, we aim to gain better understanding of the role of Pro-NT in the prediction of cardiometabolic diseases (as characterized by obesity, hyperglycemia, insulin resistance, dyslipidaemia, and hypertension) in different population-based studies.

1.1 Proneurotensin

The Neurotensin (NT) hormone was initially isolated from purified bovine hypothalamus by Carraway and Leeman in 1973. The name indicates its neuronal location as a neuropeptide and its vasodilatory actions like hypotension, inducing vascular permeability and regulation of intestinal and uterine contractions.(2) It acts as a neurotransmitter/neuromodulator in the central nervous system (CNS), as a local hormone in the digestive tract and in the cardiovascular system, and as a growth factor affecting a variety of normal or cancer cells. The subcellular cycle of NT involves a larger inactive precursor peptide of 169 amino acids named as Pro-NT, cleavage of which leads to the activation of NT.(3)

It is unknown if Pro-NT has any biological activity by itself, but it is more stable and easier to measure in plasma and therefore serves as a marker for mature NT plasma concentration.(4)

1.1.1 Neurotensin and its receptors

The physiological and pharmacological effects of NT are mediated through its interaction with three known NT receptor subtypes (NTRs) at the plasma membrane of target cells. *NTR1* (expressed from NTSR1 gene) are G-protein coupled receptors and have a high affinity for NT as determined by using a specific non-peptide NT antagonist SR 48692.(5, 6) *NTR2* are classical G-protein coupled receptors bearing seven transmembrane domains and are expressed in the brain and other extra GIT

sites. The NTR2 are recognized by the antihistamine H1 receptor antagonist levocabastine.(7) *NTR3* (*sortilin receptor*) are single intracellularly located receptors with transmembrane domain and non-coupled to G-protein.(7) There may also exist a fourth NT receptor *NTR4*, identified as *SorLA/LR11* expressed only in neurons.(8) Its exact role in the NT system remains to be determined. This heterogeneity in the structure of NTRs explain the complexity, when studying the neurotensin system and its effect, both in the brain and in the peripheral tissues.

The *NTR1* receptors mediate several central and peripheral effects of NT and plays a role in the modulation of neurotransmitter systems. A study using *NTR1* knock-out mice has implicated the important role of this receptor in weight control, feeding and thermal regulations(9) whereas NT induced pain modulation involves both *NTR1* and *NTR2*. The anti-psychotic and hypothermic effects of NT are mediated through *NTR1*.(10)

1.1.2 Neurotensin and the central nervous system

In the central nervous system, NT interacts with the dopaminergic system and induces opioid-independent pain modulation and hypothermia.(11) Experimentally, it is related to the pathophysiology of several disorders like schizophrenia, drug abuse, Parkinson's disease and eating disorders.(12) In the peripheral circulation, NT is rapidly degraded by peptidases and cannot penetrate the blood-brain barrier. However, when NT is delivered directly to the brain, it produces several physiological effects like analgesia, hypothermia and antipsychotic effects showing its therapeutic value in some medical conditions.(13)

1.1.3 Neurotensin and the gastrointestinal tract system

In the GIT, NT participates in regulating different digestive processes that take place in the ventricle, the small intestine, and the colon. Food rich in dietary fat, are major stimuli for NT release in the small intestine.(14) In humans, an increase in NT levels and a reciprocal decrease in gastric acid secretion after infusion of emulsions of fatty acids and lipids suggests a causal role of NT in inhibiting gastric acid secretions.(15) NT has also been implicated as a critical mediator of enterohepatic bile acid recycling that supports its role in fat digestion.(16) In the upper GI tract, NT reduces pressure of the lower esophageal sphincter, inhibits gastric and duodenal motility suggesting its role in regulating postprandial motility(14) whereas it stimulates defecation in the lower GIT.(17) Evidence indicates that NT is a pro-inflammatory peptide, and its effects on intestinal inflammation is mediated through *NTR1* receptors, whereas high levels of *NTR1* expression are found in human cancers from several anatomic sites including pancreatic, lung, breast, colon, colorectal and prostate cancers.(18) NT also plays an important role in mucosal regeneration,

secretory diarrhea, IBS, and inflammation induced by chemicals or bacterial enterotoxins. Moreover, high levels of NT among obese individuals is strongly linked to fat accumulation in the liver and related to both the presence and severity of Non-Alcoholic Fatty Liver Disease (NAFLD).(19) Similarly, genetically deficient NT mice absorbed less fat from the intestines and were protected from liver steatosis, insulin resistance and diet-induced obesity.(20)

1.1.4 Neurotensin and the cardiovascular system

In the heart and circulatory system, NT produces a variety of actions largely mediated via *NTR1.* This includes effects on heart rate, myocardial contractility, systemic blood pressure, coronary vascular tone, venous smooth muscle tone and regional blood flow in the GIT, cutaneous and adipose tissue. Recent studies have shown the role of sortilin gene (SORT1) for fat metabolism in the liver and in the pathogenesis of coronary artery disease in humans showing its novel role for lipid-lowering therapies and preventing myocardial infarction.(21)

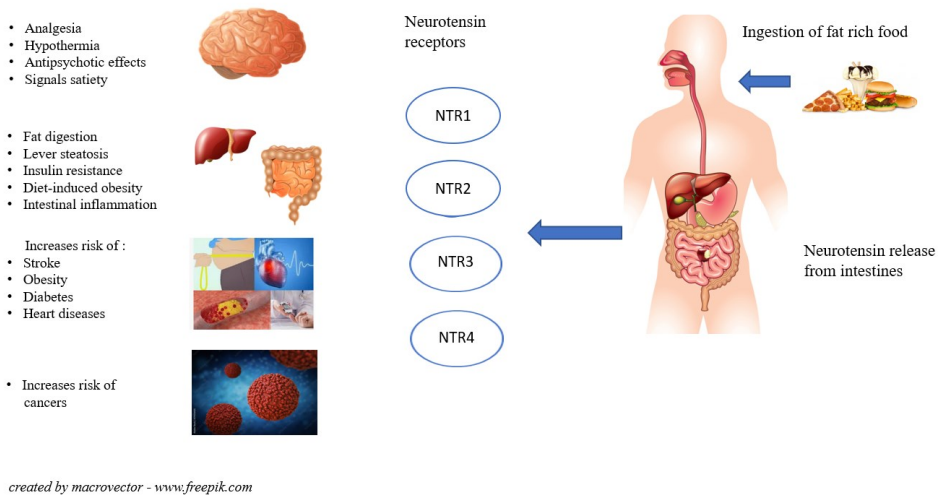

Figure 2: Proneurotensin and its effects on the human body.

1.2 Obesity

1.2.1 Definition and pathophysiology

In 2000, WHO defined obesity a state "in which excess of body fat has accumulated to such an extent that health may be adversely affected."(22)

Generally, obesity is a multifactorial energy imbalance in which calorie (energy) consumption is higher than energy expenditure, leading to an accumulation of excess body fat and weight gain. Research in recent years has improved our understanding of adipose tissue, gut and liver hormone regulations, appetite, food cravings and satiety, but has also increased our understanding of adipose tissue dysfunction causing secondary health problems.

The recent version of the International Classification of Obesity for adults by WHO is presented in Table 1.

Table 1: International classification of adult obesity based on body mass index (BMI) and risk of comorbidities. Source: From WHO 2000 and WHO 2004.(22)

Classification	BMI (kg/m²)	Risk of comorbidities
Underweight	<18.50	Low
Normal range	18.50 – 24.99	Average
Overweight	≥ 25.00	
Preobese	25.00 – 29.99	Increased
Obese class I	30.00 – 34.99	Moderate
Obese class II	35.00 – 39.99	Severe
Obese class III	≥ 40.00	Very severe

1.2.2 Global epidemiology of obesity

Obesity has become a worldwide epidemic and it is increasing dramatically in both children and adults regardless of race/ethnicity, gender, or age. The Global Burden of Disease Study reports an increased prevalence of obesity in developed and developing countries over the last three decades. It has increased from 3% in 1975, to 11% among men and from 6% to 15% among women in 2016.(23) Prevalence rates of obesity in men and women are heterogeneous across different parts of the world. Men have higher rates of overweight and obesity in the developed countries whereas women have higher rates in the developing countries. These differences reflect not only an obesity transition across subpopulations within the countries but also the socioeconomic factors, exposure to obesogenic foods, and local environmental factors. Furthermore, it also includes the risk factors that affect individual's physiology, psychology, physical activity, food production and food consumption.(24)

1.2.3 Is obesity a disease?

Obesity increases the risk of metabolic diseases (for example T2D and fatty liver disease), CVD (myocardial infarction, and stroke), musculoskeletal disease (osteoarthritis), Alzheimer disease, depression, some type of cancers (for example, breast, ovarian, prostate, liver, kidney, and colon cancer) and premature mortality.(25)

On a basic level, it is a condition where calorie (energy) consumption is higher than energy expenditure. However, besides this, powerful homeostatic mechanisms are found in obese people that hinder weight loss and promote further weight gain. These altered biological mechanisms explain why short-term radical changes in lifestyle factors and medical interventions are usually not sufficient enough to accomplish long-term weight loss.(26)

1.2.4 Why does not everybody develop obesity?

The mechanisms that define the susceptibility of an individual to obesity in an obesogenic environment are still not clear. “Western diet”, containing high levels of sugars and fats and low levels of dietary fibres, is clearly an important driver of the obesity pandemic, but everybody does not develop obesity under the same environmental conditions. Heritable genetic (i.e., the *FTO* gene) and/or epigenetic factors as well as behavioural factors are suggested to be important moderators of energy balance. The bimodal disease model suggests that epigenetic mechanisms usually ‘switch on’ obesity, but only in some people while other people remain protected.(26, 27) Another concept to explain the BMI heterogeneity among humans is the genetically determined susceptibility differences that exist in the central control of food intake mediating the response to overeating.(28) Similarly, monogenetic causes underlying BMI heterogeneity are rare to explain the obesity pandemic, but combinations of small individual contributions of many genetic obesity risk loci along with the long-term effects of obesogenic environmental factors might provide an explanation to the obesity epidemic.

1.2.5 Metabolically healthy obesity

Epidemiological studies have described subgroups of obese individuals with a normal metabolic profile and lower risk of obesity-related cardiometabolic complications than other obese subgroups with a high risk of such complications. This subgroup has been identified as having “metabolically healthy obesity” and accepted criteria include absence of abdominal obesity, absence of components of metabolic syndrome, normal insulin sensitivity and a high level of cardiorespiratory fitness. This indicates that substantial heterogeneity exists in the individual responses to obesity.(27) However, it is still questionable whether metabolically

healthy obese individuals stay healthy. Some studies have shown that metabolically healthy obese individuals have an increased risk of heart failure despite significantly lower risk of cardiovascular disease and several psychological and social factors, chronic pain, cancer, and other conditions.(28)

1.3 Dyslipidaemia

Dyslipdaemia covers a broad spectrum of disturbances in lipid metabolism resulting in changes in plasma lipoprotein functions and/or levels. It can be related to other diseases (secondary dyslipidaemia) or due to the interaction between genetic predisposition or environmental factors. Evidences from multiple randomized controlled trials (RCTs) show that reduction of total cholesterol and low-density lipoprotein cholesterol (LDL-C) can prevent atherosclerotic cardiovascular disease.(29) Brown & Goldstein discovered the molecular mechanisms of atherosclerosis by explaining the progressive accumulation of cholesterol in the plasma and arteries due to a defect in the LDL receptors, resulting in familial hypercholesterolemia.(30)

Atherosclerotic lesions begin with the dysfunction of vascular endothelium. This is followed by recruitment and activation of monocytes to macrophages in the arterial wall. These macrophages take up the cholesterol and other lipids from the lipoproteins and become foam cells. These lipid-rich foam cells, together with the proliferation of vascular smooth muscle cells, cause fatty streaks in the arterial wall.(31) Vascular atherosclerosis seen in patients with T2D depends not only on glycemic control but also on the duration of the disease and the presence of co-existent risk factors like obesity and hypertension. Intensive glucose control strategies in intervention studies like the *Actions in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial* and *the Outcome Reduction with Initial Glargine Intervention (ORIGIN) trial* did not show evidence of reduction in macrovascular events like death from CVD causes, nonfatal myocardial infarction or nonfatal stroke.(32, 33)

In recent years, a post-alimentary rise of triglyceride-rich lipoprotein (TRL) in the blood after a fat-rich meal resulting in postprandial lipidaemia is implicated in the increased risk of premature atherosclerosis, progression of CVD and other metabolic conditions such as obesity, diabetes, and the metabolic syndrome.(34, 35) Similarly, the statin therapy aimed at lowering LDL cholesterol reduces the cardiovascular events by only one third, whereas two-third of the “residual risk” is proposed to be dependent on the plasma remnant lipoproteins in the blood.(36) Lowered HDL, raised plasma TG and TG-rich lipoproteins (TRL) including remnant chylomicrons, VLDL, and small dense lipoprotein (sd-LDL) are known to

constitute an atherogenic and pro-inflammatory lipid profile or atherogenic lipid triad that contributes to the risk of CVD in diabetic dyslipidaemia.(29, 37)

Poor dietary quality was one of the leading mortality risk factor contributing to 11 million deaths and half of the CVD deaths globally among aged 45-65 years (1999-2017).(38) Postprandial lipemic response can be reduced by dietary choices and depends on the fatty acid composition of the food. Saturated fatty acids (SFA) are rich in foods containing animal fats and causes more pronounced lipemia than monounsaturated fatty acids (MUFA), e.g., olive oil. Similarly, polyunsaturated fatty acids (PUFA) in vegetable and fish oils have been suggested to result in the lowest postprandial rise of lipidemia.(39) Vascular inflammation in the post-prandial state is modulated by the effects of TRL and effects of different dietary fats on the macrophage signaling pathways. This emphasizes the role of different dietary fats in the regulation of vascular functions.(40)

1.4 Cardiovascular diseases

1.4.1 Epidemiology of cardiovascular diseases

CVD includes myocardial infarction (MI), angina, stroke, and peripheral vascular disease and individuals without revascularization procedures. These are the leading causes of death worldwide and a major contributor to the disability. In the last 2-3 decades, age-standardized CVD deaths steadily decreased in high-income countries, whereas this only slightly decreased or even increased in most of the low- and middle-income countries primarily due to better preventive interventions and optimal management of diabetes, blood pressure, and dyslipidaemia.(41) According to the recent updates from "Global burden of CVD" 2019 study, the global trends of disability-adjusted life years were higher in men before the age of 80-84 years and in women at age >80 years. This implicates a need of further attention to cause-specific mortality at older ages but even to develop strategies for CVD prevention(42)

Most complex multifactorial CVD studies in the latter half of the 20th century were prospective population-based studies. In the Framingham Heart Study, risk factors predisposing to coronary heart disease (CHD) were identified. Additionally, criteria of heart failure and risk factors for atrial fibrillation were specified and a multivariate model of the 10-year CHD risk was developed known as the "*Framingham Heart Score*."(43) In the follow-up study after 44 years, high risk of hypertension and cardiovascular diseases (angina pectoris, myocardial infarction, coronary artery disease or stroke) were observed in both overweight and obese men and women, aged 35 to 75 years.(44)

1.4.2 Risk factors and comorbidities for CVD

In 1961, the term "***risk factor***" was defined by Kannel as, "*any qualitative or quantitative variable in patients, which is statistically significantly associated with increased incidence of, or mortality due to specific disease*."

Determinants of the CVD can be modifiable or non-modifiable factors. Some of the non-modifiable determinants are age, gender, family history (heredity) and ethnicity whereas modifiable determinants of CVD are smoking (tobacco use), obesity, unhealthy diet, physical inactivity, and regular alcohol intake etc. Prevention of the disease can take place before the occurrence of the disease (primary prevention) or after the first presentation (incidence) of the disease (secondary prevention).

Some of the preventions are primordial in which health factors are promoted and facilitated on a group level, e.g., limiting the sale of alcohol or banning smoking. It is very important to determine if the proposed risk factor/marker is the real cause of the disease. For example, use of antihypertensive treatment is associated with an increased risk of CVD in observational studies, but it doesn't cause the disease. These individuals on antihypertensive drug treatment already have a vascular system affected by hypertension. This is an example of reverse causation.

Figure 3: Risk factors/ comorbidities associated with the development of cardiovascular diseases

1.5 Hypertension

According to The Task Force for European Society of Cardiology (ESC) and the European Society of Hypertension (ESH), hypertension is defined as, "*systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) values ≥ 90 mmHg*".(45)

High blood pressure is one of the important modifiable risk factors of CVD mortality and disease burden. In 2015, around 8.5 million deaths were attributed to SBP > 115mmHg, of which 88% were in low-income and middle-income countries. According to NCD-RISC data in 2015, there was a decline in mean systolic blood pressure of the population despite a rise in BMI in high-income countries, which is an established risk factor for hypertension. Similarly, men had a higher SBP and DBP than women in most countries except sub-Saharan Africa and few countries in Oceania and Asia, where this sex-specific pattern was reversed.(46)

The Framingham Heart Study showed an increased risk of CVD associated with high normal blood pressure (130-139 mmHg for SBP and 85-89 mmHg for DBP) as compared to optimal blood pressure (< 120 mmHg for SBP and < 80 mmHg for DBP) or both. Furthermore, 90% of normotensive individuals at age of 55 years, later developed hypertension showing an age-associated increased prevalence of hypertension.(47)

The Action to Control Cardiovascular Risk in Diabetes Blood Pressure study (ACCORD-BP) did not demonstrate a significant benefit of intensive blood pressure lowering on the primary composite endpoint (myocardial infarction, stroke, and CVD death).(48) On the other side, the effect of Systolic Blood Pressure Intervention Trial (SPRINT) showed reductions in CVD events in hypertensive patients without T2D when randomised to SBP <120 mmHg vs. <140 mmHg.(49-51)

The most recent guidelines from American Diabetes Association and the European Society of Cardiology recommend an SBP target <130 mmHg or lower if tolerated but not lower than 120 mmHg in patients with T2D. Target SBP range should be 130-140 mmHg in older patients (aged ≥ 65 years). DBP target should be < 80 mmHg but not < 70 mmHg.(52, 53)

1.6 Type 2 Diabetes

1.6.1 Risk factors and pathophysiology

T2D is a metabolic disorder caused by impaired insulin secretion and insulin resistance resulting in decreased glucose utilization by the tissues, excessive hepatic glucose production, hyperglycaemia, and low-grade systemic inflammation. Prospective studies have shown strong associations of T2D with central obesity, dyslipidaemia, hypertension, increased accumulation of fat in the liver (hepatic steatosis) resulting in a series of complications such as CVD including angina, stroke, myocardial infarction, and coronary artery disease, but also neuropathy, blindness, kidney failure, cognitive decline, and premature death. T2D has a long preclinical phase and 30-50% of individuals remains undiagnosed for many years.

The pathophysiology of T2D includes insulin resistance and β-cell dysfunction that promotes a pro-inflammatory state and dyslipidaemia resulting in atherosclerotic plaque progression whereas chronic hyperglycaemia interferes with multiple metabolic pathways resulting in microvascular complications (e.g., retinopathy, neuropathy, and nephropathy). However, the failure of β-cells to produce insulin is the critical step for pre-diabetes evolving into overt T2D.(53) Prediabetes is defined as impaired fasting glucose and/or impaired glucose intolerance. In the prediabetic stage, insulin secretion is increased initially to compensate for the insulin resistance, but subsequently the insulin secretion decreases due to further β-cells dysfunction resulting in increased blood glucose.(54) Correspondingly, most genes associated with T2D act on insulin secretion, but only a minority are of importance for insulin sensitivity.

Risk factors/markers of T2D includes a combination of both genetic and metabolic factors, where non-modifiable factors include ethnicity, a first-degree family history of T2D, being older than 40 years of age or in women having a history of gestational diabetes. Modifiable factors include obesity, a sedentary lifestyle, unhealthy diet, physical inactivity, and smoking. Prevention of disease progress in high-risk individuals can be achieved by lifestyle changes including improved diet and increased physical activity levels, pharmacological interventions, and bariatric surgery in severe obesity. Individuals with varying degree of impaired glucose tolerance and diabetes are shown in **Table 2**.

Table 2: Oral glucose tolerence test and HbA1c indicators for Prediabetes and Diabetes according to American Diabetes Association.(55)

Impaired fasting glucose (Prediabetes): Fasting P – Glucose: 5.6 – 6.9 mmol/L and/or 2 hours P – Glucose < 7.8 mmol/L
Impaired glucose tolerance: Fasting P – Glucose: < 6.1 mmol/L and/or 2 hours P – Glucose: 7.8 – 11 mmol/L
Impaired glucose regulation: Fasting P – Glucose: 6.1 – 6.9 mmol/L and/or 2 hours P – Glucose: 7.8 – 11 mmol/L
Diabetes Mellitus: Fasting P – Glucose: ≥ 7.0 mmol/L and/or 2 hours P – Glucose ≥ 11.1 mmol/L
Diabetes Mellitus: A1C ≥ 48 mmol/mol.

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1.6.2 Type 2 diabetes and obesity

The prevalence of T2D is rising due to improved medical treatments and declining mortality globally.(56) The prevalence of T2D is expected to rise from 8% in 2011 to 10% in 2030. In 2019, around 1.5 million deaths are attributed to T2D and 48 % of all deaths due to T2D occurred before the age of 70 years.(57) In the Framingham Heart Study, T2D incidences remained highest among the obese individuals over the four decades (1970-2000).(58) In the Health Professional Follow-up Study, a tenfold increased risk of developing T2D was observed in men with BMI >35kg/m² and in women with BMI >29 kg/m².(59)

Among existing T2D incidence data, a few studies report a fall or stabilisation of T2D incidence in the last decade in Europe and East Asia, but data are limited in low and middle income countries. In Sweden, a rise in the prevalence of pharmacologically treated T2D was reported from 2006 to 2014, however, the incidence of T2D is decreasing. Strategies for prevention of T2D and public health education are some of the contributing factors to this falling incidence.(60)

1.6.3 Type 2 diabetes and cardiovascular diseases

High glucose levels over time play an important and independent role in the development of CVD. Cardiovascular pathologies of T2D include abnormalities in systemic and local vascular inflammation, endothelial and microvascular injury, altered thrombosis, autonomic dysfunction and most likely membrane instability in nerves, smooth muscle cells and endothelium. Insulin resistance and metabolic changes in T2D accelerates the development of atherosclerosis whereas diabetic cardiomyopathy results in diastolic dysfunction, ventricular hypertrophy, and cardiac remodeling even without coronary artery disease.(61)

The risk of developing cardiovascular complications of T2D after exposure to high glucose levels for a certain period is called as “metabolic memory” or “legacy effect”. This results in the formation of advanced glycation end-products (AGE) due

to high blood glucose levels and are not easily metabolized. These accumulations accelerate the progression of vascular disease in the diabetic patients.(62)

Intensive glucose control in the trial, *Action in Diabetes and Vascular Disease-PreterAx and DiamicroN Controlled Evaluation* (ADVANCE) resulted in the reduction of microvascular complications; however, no significant effects were observed on the risk of major macrovascular complications like coronary artery disease, or peripheral vascular disease (63) Recently, T2D patients with high risk of cardiovascular events had significantly lower rate of primary composite cardiovascular outcome and death if treated with sodium and glucose transport- 2 (SGLT-2) inhibitors, for example in the study, *Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients* (EMPA-REG).(64) Correspondingly, treatment with glucagon like peptide 1 (GLP-1) receptor agonists/analogues resulted in a lower risk of the primary cardiovascular outcome and death from cardiovascular diseases, as observed in the trial, *the Liraglutide Effects and Action in Diabetes: Evaluation of cardiovascular outcome Results* (LEADER).(65) These studies emphasize the benefits of cardiovascular safety with new T2D therapies, of special benefit to patients at high or very high cardiovascular risk (secondary prevention).

1.6.4 Type 2 diabetes and dyslipidaemia

T2D and atherosclerosis are interlinked through several pathological pathways. Dyslipidaemia with high levels of atherogenic LDL cholesterol, hyperglycemia, increased oxidative stress, and associated with increased inflammation are some of the proposed mechanisms. Plasma LDL is currently recognized as the main source of lipid in the plaque, but studies have shown that native LDL particles by themselves do not cause intracellular lipid accumulation. It is the atherogenic modification of LDL, which alters the physical and chemical characteristics of LDL particles, which leads to lipid accumulation and atherosclerosis.(62)

1.7 Migration – A public health perspective

We are living in a world where around 260 million people are migrants, living outside their country of birth. The relationship between migration and health is multifactorial and an important contributing factor in determining global health.

European countries are one of the main recipients of international migration in the last decades. Prevalence of T2D has also moderately increased in Europe and the age-adjusted prevalence rate is expected to rise from 6.3% in 2019 to 7.8% in 2045. Epidemiological studies have shown that migrants constitute a vulnerable group for T2D as they are exposed to variety of adverse effects in the pre-migration, migratory

and post- migration phases, resulting in an increased risk for both physical and mental health and leading to worse health outcomes.(66)

1.7.1 Disease risk and ethnicity

Collection and study of race and/or ethnicity data is an important way to identify relevant demographic health outcome differences and to address the disparities in health care provision and treatments. The American Medical Association (AMA) defines ethnicity as follows: *"Ethnicity relates to groups of people classed according to common racial, national, tribal, religious, linguistic, or cultural origin or background. Like gender, race and ethnicity are cultural constructs, but they can have biological implications."*(67) Differences in immigrants health and health related outcomes between different ethnic groups are well-known and studied. Researchers in countries like Sweden, Denmark, Germany, Norway, and UK have compared immigrant groups to the native populations for a variety of outcomes. An apparent example is the comparison of CVD risk factors between German and Norwegian natives with immigrants from the Middle East (Turkey, Iran, Pakistan) which showed that native population have higher blood pressure and total cholesterol levels but lower total triglycerides (p-TG) and higher high-density lipoproteins (p-HDL) levels.(68) During the past decades, 400 genetic variants known as Single Nucleotide Polymorphisms (SNPs) have been identified to modulate the risk of T2D through insulin secretion, either through a direct effect on islet cells function (e.g. *KCNJ11*) or islet development (*HNF1A*), or indirectly through impact on incretin signalling (*GLP1R*). Still additional work is required to identify causal variants/genes that confer T2D risk in various ethnic groups.(69) Similarly, epigenetic factors i.e., modifying effects of behaviour and environment on the genes and gene activity only partly explains the increased cardiometabolic risk in certain population groups.(70)

1.7.2 Type 2 diabetes risk and Immigrants to Sweden

Studies conducted in Sweden during the last two decades have shown that the prevalence of T2D among non-European immigrants was 2-3 times higher as compared to people born in Sweden.(66, 71) In 2010, Wändell *et al*. reported a higher T2D risk among non-European immigrants than in native Nordic populations with the highest risk being observed in women.(71) In the MEDIM study, immigrants from Middle Eastern countries living in Southern Sweden have higher prevalence rate of T2D. It was shown that they are more insulin resistant and have higher HbA1c levels as compared to native Sweden-born population in the non-diabetic stage. On the contrary, lower prevalence of hypertension and better renal function in Iraqi-born individuals as compared to native Swedes are also observed.(72, 73)

Various factors attributes to this high T2D risk in immigrant populations such as socioeconomic factors like low education and unemployment, environmental factors like physical inactivity, sedentary lifestyle, and poor dietary habits. Increased prevalence of known risk factors for cardiometabolic disease like obesity, family history of T2D, abdominal obesity and T2D onset at an early age are also observed in immigrant population.(74) Furthermore, a high genetic susceptibility to T2D has been reported in some immigrant groups.(75) However, all these contributing factors cannot fully explain the high risk of T2D in Iraqi immigrant populations and thus need further studies.

2 Aims

The overall aim of this thesis was to investigate the effects of Pro-NT hormone on the risk of obesity, cardiometabolic diseases and mortality in the general population, and to determine if Pro-NT is a useful biomarker.

The specific aims in the subprojects were:

I. To assess if fasting levels of Pro-NT predicts CVD and T2D development in an elderly population.

II. To study the modifying effects of Pro-NT on glucose regulation in an immigrant population of Sweden.

III. To explore the acute effects of a standardised oral lipid load on Pro-NT and plasma triglycerides levels in healthy individuals.

IV. To identify the predictive value of Pro-NT on all cause and cause-specific mortality in a middle-aged cohort.

3 Subjects and Methods

3.1 Study cohorts and populations

3.1.1 The Malmö Preventive Project

The Malmö Preventive project (MPP) was a prospective population-based cohort, started at the Section of Preventive Medicine, Department of Medicine, University Hospital, Malmö, in 1974. The main aim of the study was to find high-risk individuals for preventive interventions on cardiovascular risk factors, alcohol abuse, impaired glucose tolerance and screening of breast cancer. Broad health screening programs, including self-administered questionnaire for medical and personal history, a physical examination, and a panel of laboratory tests were provided. Participants were defined as those of the invited subjects who attended the screening examination and born in pre-specified years. In all, 33,346 mostly middle-aged men (n= 22,444) and women (n=10,902) participated between 1974 and 1992, representing an overall attendance rate of 71.2% (range 64-78%). Men were mostly screened during the first half of the period (1974-1982) and women during the latter half (1981-1992), thus implying different follow-up periods for men and women. Mean age at baseline was 44 years for men (27-61 years) and 50 years for women (28-58 years). Most of the participants were white Europeans living in Malmö.(76, 77) Data from the MPP was used for the analyses in ***Paper I.***

3.1.2 MPP Re-examination

Between 2002 and 2006, a total of 18,240 individuals of the MPP who were still alive and had not migrated from Sweden, attended a follow-up re-examination including anthropometric measurements, a questionnaire on lifestyle factors and blood samples. A random sample of 5402 participants with fasting blood samples were selected for the *study I* of this thesis. Prior participation in MDC Study (1992-1996) was one of the exclusion criteria to obtain non-overlapping cohorts. Before the baseline of the current study, 578 individuals with prevalent CVD diagnosis in the analysis of incident CVD, and 640 individuals with prevalent T2D diagnosis in the analysis of incident T2D were also excluded. After further exclusion of subjects

with missing data of any covariate, 4804 subjects were included in the analysis of incident CVD and 4511 subjects in the analysis of incident T2D.

The health service Authority of Malmö approved and funded the screening program. The regional ethics committee at Lund University approved the linkage of participants ID with the cause of death and patient registers. (LU 2004/85; and LU 2011/412).

Written consent was not available for MPP when study was conducted in 1974, however all participants gave written informed consent in MPP Re-exam study.

Flowchart of the participants in the study are shown in **Figure 4**.

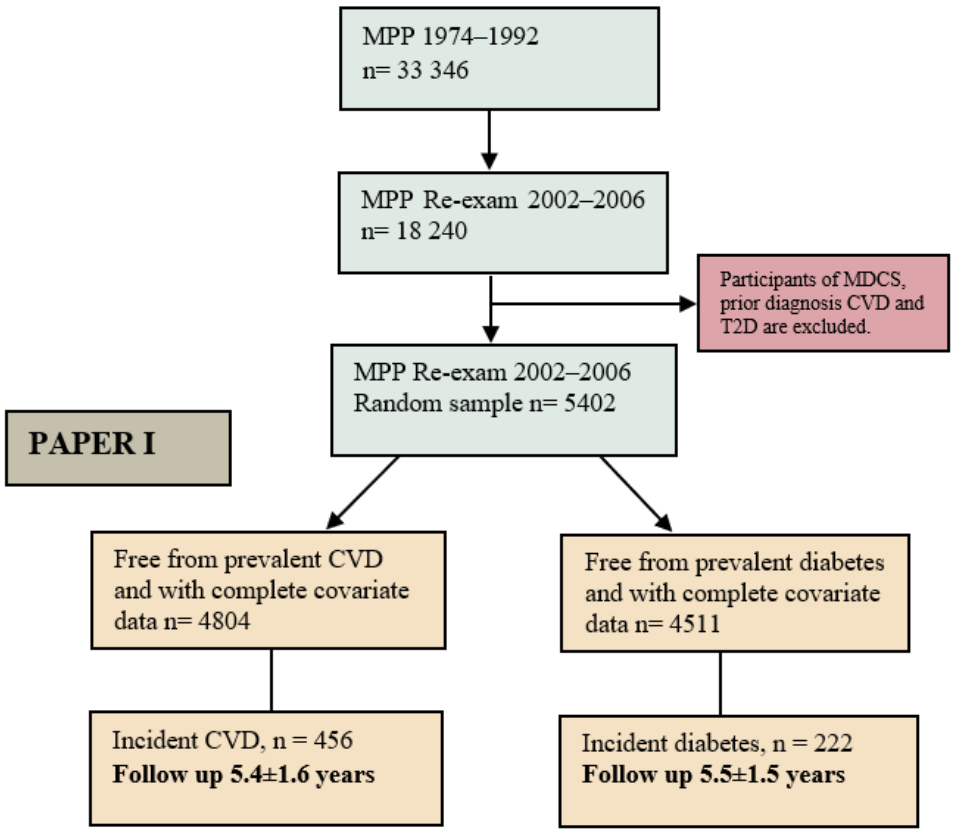

Figure 4: Flow chart to represent recruitment of participants in the Malmö Preventive Project, baseline, and Re-examination.

3.1.3 The MEDIM study

The MEDIM (impact of Migration and Ethnicity on Diabetes in Malmö) study is a population-based, cross-sectional cohort of men and women, 30-75 years of age, born in Iraq (n=1398) or in Sweden (n=757). The cohort was sampled between 2010 and 2012 in Malmö, a city in the southern Sweden. Participants constituted of two groups: (a) Those born and living in Sweden, and (b) those born in Iraq (first-generation immigrants) and living in Sweden. All participants were living in the same geographical area and matched for age and sex distributions. Participants with type 1 diabetes, severe physical disabilities, or mental illness were excluded from the study.(78) Data from the MEDIM study was used in analyses in ***Paper II.***

Ethical permissions for the MEDIM study were obtained from the Lund University ethical review board. (ID LU 2009/36 and 2010/561).

The study was compiled in accordance with the Declaration of Helsinki and all participants provided written informed consent.

Flowchart of the participants in the study are shown in **Figure 5.**

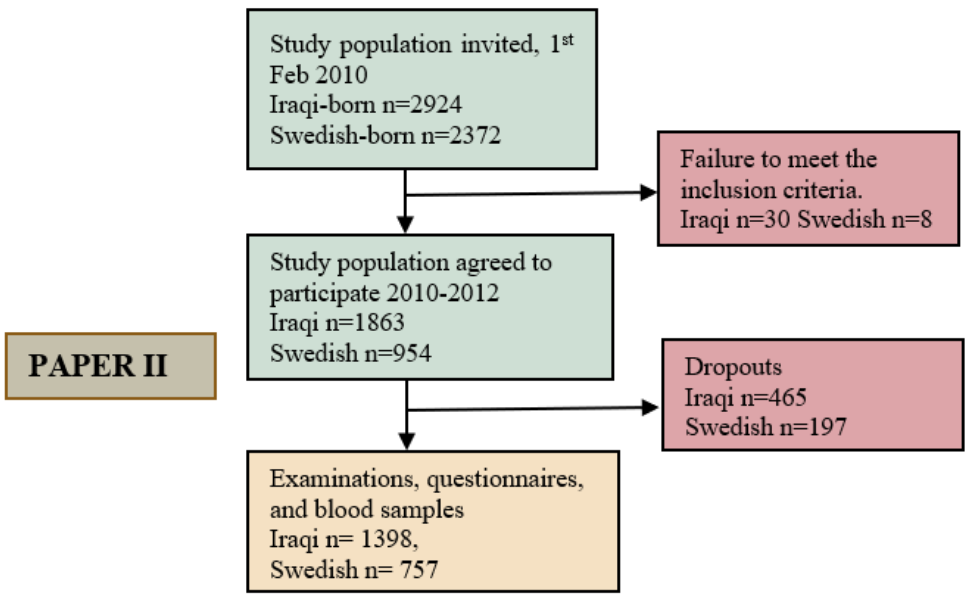

Figure 5: Flow chart to represent recruitment of participants in the MEDIM study.

3.1.4 The Malmö Diet and Cancer Study

MDCS was conducted between March 1991 and October 1996 in collaboration with the International Agency for Research on Cancer (IARC) Lyon, France, The Swedish Cancer Society and the Faculty of Medicine, Lund University, Sweden. The main aim of this study was to investigate the relationship between diet, life-style factors, and prospective risk of various forms of cancers.(79, 80) All men born between 1923 and 1945 and all women born between 1923 and 1950 that were residents of the city of Malmö at the time (n= 74,138) were invited to participate in the study. Two means of recruitment were used in parallel combining active recruitment by personal invitation letters and passive recruitment directed towards the whole community by using advertisements in public places and local newspapers between January 1991 and September 1996.(81) The exclusion criteria for recruitment were mental incapability and difficulties with Swedish language skills. A total of 30,447 invited participants responded and were initially included. A total of 28,449 (11,246 men and 17203 women) attended the baseline examinations and provided written informed consent corresponding to a participation of 40.8% (for men 38.3% and for women 42.6%).(80, 81)

Each participant visited the study centre twice. During the first visit, anthropometric measurements (weight, height, waist and hip circumference and BMI) were carried out, and all participants filled questionnaire with addressing dietary habits, lifestyle and socioeconomic background including details of smoking, alcohol, previous weight changes, previous and current diseases, and use of medications etc. Blood pressure was measured and trained nurses collected non-fasting blood samples stored in the biological bank at -80 ºC.(82) These dietary surveys were discussed in detail at individual interviews with a nutritionist during the second visit within two weeks. (Fig. 6)

3.1.5 The Malmö Diet and Cancer Study-Cardiovascular Cohort

Of the MDCS participants, enrolled between October 1991 and February 1994 (n=12,445), approximately 50% (n= 6,103) were randomly selected and invited to participate in an ultrasound screening study of carotid artery morphology forming a sub-cohort, the MDC-CC. The reason for non-participation were either sickness, emigration (n= 143), lack of fasting samples (n= 292) or death (n= 1036). These participants provided additional data on their medical history, an additional physical examination, and fasting blood samples for measurement of fasting glucose, fasting serum lipid and lipoprotein concentrations. (83, 84) In ***Paper IV***, data from the MDC-CC was used for the analyses.

Ethical permissions for the studies were obtained from the Lund University ethical review board ID (LU 5190 and 652/2005).

The study was compiled in accordance with the Declaration of Helsinki and all participants provided written informed consent.

Flowchart of the participants in the study are shown in **Figure 6**.

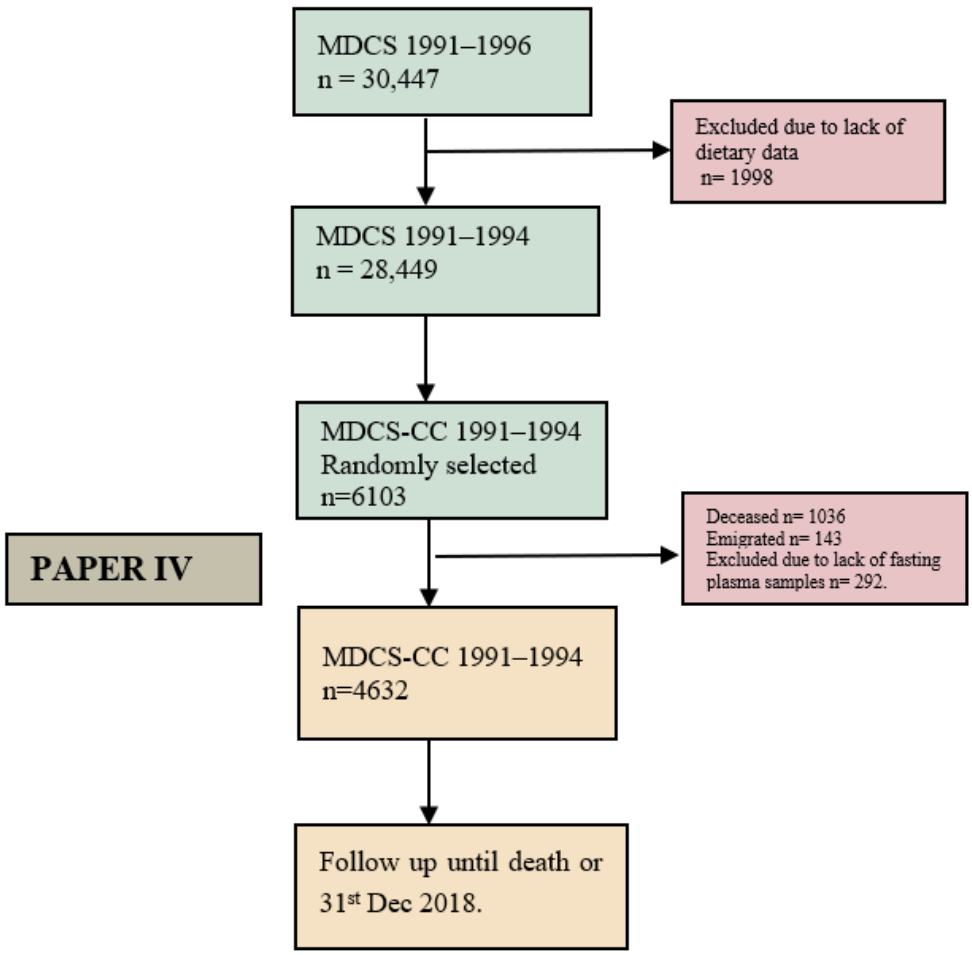

Figure 6: Flow chart to the recruitment of partcipants in MDCS and MDCS-CC.

An overview of the studies included in this thesis is presented in **Table 2.**

Table 2: Summary of studies included in this thesis.

	MPP study	**MEDIM study**	**Oral lipid load study**	**MDCS-CC study**
Papers	I	II	III	IV
Design	Prospective cohort study	Population-based cross sectional study	Human experimental study	Prospective cohort study
Participant recruitment	2002-2006	2010	2018-2019	1991-1994
Participants	CVD, n= 4804 T2D, n= 4511	Born in Sweden n=757 Born in Iraq n=1398	n= 22	n= 4632
Main Outcomes	Incidence CVD Incidence T2D	Prevalence & risk of T2D	Prevalence of hypertriglyceridemia	All-cause mortality & cause-specific mortality.
Statistical Analysis	Cox regression, Kaplan-Meier curve	Linear model, logistic regression	Paired t -tests Pearson correlation analysis	Cox regression, Kaplan-Meier curve
Follow-up time	CVD = 5.4 ± 1.6 years T2D = 5.5 ± 1.5 years			20 ± 3 years

3.2 Assessment of exposures /predictors

Blood samples from each participant were fractionated, i.e., 10 ml blood without anticoagulant was used to store serum sample (at -80 º C) and 28 ml of heparinized blood for leucocytes (at -140 º C) and plasma (at -80 º C) for later analyses. Data on cardiovascular risk factors/markers were reassessed, and fasting plasma samples were drawn and immediately stored at – 80 º C for later analyses.(85, 86)

As mature neurotensin has a short half-life, a stable precursor fragment of neurotensin (Pro-NT) was measured in the previously stored fasting plasma. One-step sandwich immunoassay based on a chemiluminescence label and coated-tube technique were used (SphingoTec©, Hennigsdorf, Germany) with 1.9 pmol/l as the limit of detection of Pro-NT precursor fragment (Pro-NT 1-117).(87)

3.3 Assessment of covariates

3.3.1 Paper I & Paper IV

*Data from MPP was used for **Paper I** and MDCS-CC was used for **Paper IV**.*

In ***Paper I***, fasting samples of 5402 participants were selected for the first study of this thesis based on the available fasting blood samples. In ***Paper IV***, 4632 participants were included.

Self-administered questionnaire gathered information about age, sex, family history of diabetes, hypertension and CVD, current use of antihypertensive or antidiabetic drug medications, cigarette smoking habits, physical activity (habits), alcohol use, history of malignancies, and level of education. Participants were classified into four categories regarding cigarette smoking: (current smokers, occasional smokers, ex-smokers, or non-smokers).

Anthropometric measurements were carried out by trained nurses with the subjects wearing light indoor clothes and without shoes. Standing height (centimeters) was measured with a fixed stadiometer. Weight (kilograms) was measured by using a calibrated balance beam scale. BMI was calculated as weight (kilograms) divided by the square of height meters. Blood pressure (mmHg) was measured twice using an oscillometer device after 5 minutes of rest in the supine position. At the clinical examinations during baseline, participants underwent a physical examination, filled a questionnaire, and provided blood samples that were stored in minus -80 º C for later analyses.

Serum cholesterol and triglycerides were analysed using enzymatic methods. HDL-cholesterol in serum was measured enzymatically after isolation of VLDL and LDL. LDL-cholesterol was estimated using Friedewald's formula. Serum insulin levels were detected using the radioimmunoassay technique. All blood samples were analysed according to the standard procedures at the Department of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden.

3.3.2 Paper II

*Data from MEDIM study was used in **Paper II.***

In ***Paper II***, fasting blood samples of 2155 subjects (1398 Iraqi- born and 757 Swedish-born) were collected for this study. Fasting plasma glucose levels, serum insulin levels, plasma triglycerides, p-HDL, and p-LDL-C levels were analysed according to the standard procedures at the Department of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden. HbA1c was measured by ion exchange chromatography.

Arabic- and Swedish- speaking research nurses gathered information by using validated questionnaires related to participants socio-economic situation, lifestyle habits, smoking habits, use of antidiabetic medications, antihypertensive treatment, and family history of diabetes (in biological parents and/or siblings). These questionnaires were further translated by two independent professional translators with Arabic as their native language. Clinical examinations for the control of blood pressure, height, weight, waist circumferences and BMI were performed by the trained nurses.

Oral glucose tolerance tests (OGTTs) were used to measure the body's response to glucose. It can reveal diabetes, insulin sensitivity, impaired glucose-stimulated insulin secretion, which reflects impaired beta cell function as well as insulin resistance. Participants were instructed not to eat or drink after 22:00 p.m. Fasting blood samples were collected followed by an oral glucose tolerance test (OGTT) where participants received 75-g of glucose. Blood samples were collected at 0, 30, 60, 90 and 120 min for the measurement of plasma glucose and serum insulin levels (assessed by using radioimmunoassay). Glucose and Insulin values obtained during an OGTT were used for the assessment of insulin sensitivity and insulin secretion as Matsuda indices.(88, 89)

The *insulin sensitivity index* (ISI) was calculated from the OGTT according to the formula:

$$\frac{10{,}000}{\sqrt{[(\text{fasting glucose (mmol/l)} \times \text{fasting insulin (mIE/l)}) \times (\text{mean OGTT glucose conc (mmol/l)} \times \text{mean OGTT insulin conc (mIE/ l)})]}}$$

Beta cell function assessed as *Corrected incremental insulin response* (CIR) was calculated by using the following formula:

$$\frac{100 \times \text{insulin at 30 min (mIE/l)}}{[\text{glucose at 30 min (mmol/l)}] \times [\text{glucose at 30min (mmol/l)} - 3.89]}$$

Disposition index represents the insulin secretion (beta cell function) adjusted for insulin resistance (insulin sensitivity) and it was calculated as a product of CIR and ISI:

$$DIo = CIR \times ISI.$$

3.3.3 Paper III

Data for ***Paper III***

In ***Paper III***, 22 healthy subjects (31± 9.5 years) participated. All subjects underwent ingestion of 150 ml of milk cream (54g of fat) and 59 ml of pure olive oil (54g of fat), at two separate occasions with one week apart. Blood samples were collected for the measurement of plasma triglycerides, glucose, and Pro-NT in the fasted state (0 hour) and at every hour for four hours.

Again, information about smoking habits, use of antidiabetic drugs, antihypertensive drug treatment and family history of diabetes and CVD were gathered using questionnaires. Blood pressure, height, weight, were measured by the trained nurses, and BMI calculated.

3.4 Assessment of outcomes

3.4.1 Paper I - IV

Endpoint ascertainment

In ***Paper I***, the primary outcome was first incident CVD (follow-up 5.4±1.6 years) and first incident T2D (follow up 5.5±1.5 years) until 31st December 2010. During the follow-up, the events were defined by linking a 10-digit personal identification number, available for all Swedish citizens, with national registers with 100% coverage and validity:

1. The Swedish Outpatient Register.(90)
2. The Swedish Hospital Discharge Register has been operating in the south of Sweden since 1970 and become nationwide in 1987.(91, 92)
3. The Swedish Cause of Death Register.(93)

For identification of incident CVD, myocardial infarction was defined based on International Classification of Diseases (ICD) 9 code 410 or ICD-10 code I21. Death attributable to ischemic heart disease was defined as ICD-9 codes 412 and 414, or ICD-10 codes I22, I23, or I25. Stroke was defined using ICD-9 codes 430, 431, 434 and 436 and ICD-10 codes I60, I61, I63, and I64.

Incident T2D were identified by linkages to six different national and regional diabetes registers. Individuals could be registered as having a diagnosis of T2D in the following registers:

1. The Swedish Patient Register, including hospital discharge register.(91)
2. The Swedish National Diabetes Register (NDR), which has been operating since 1996 and require a physician's diagnosis of all new cases of diabetes according to established diagnostic criteria.(94, 95)
3. The Regional Diabetes 2000 register of the Scania region of which Malmö is the largest city.(96)
4. The Swedish Cause of Death Register which comprises of all the deaths among Swedish residents occurring in Sweden or abroad.(93, 97)
5. The nationwide Swedish Prescribed Drug Register has been operating since 2005 to identify patients who had been prescribed insulin or anti-diabetic drug medication.(98)
6. The Malmö HbA_{1c} register (MHR), where subjects were considered to have developed T2D if they had at least two HbA_{1c} recordings ≥ 42 mmol/mol (6.0%) based on the Swedish Mono-S-based standardization system (corresponding to 53 mmol/mol [7.0%], according to the U.S National Glycohemoglobin Standardization program). All glycated haemoglobin (HbA1c) values collected from individuals in institutional and non-institutional care in Malmö from 1988 onwards were analysed at the Department of Clinical Chemistry, Malmö University Hospital.

In ***Paper II*****,** the main outcome was elevated level of plasma Pro-NT in the Iraqi-born individuals as compared to the Swedish- born individuals and its association with the indices of glucose regulations (assessed as insulin action, insulin secretion and HbA_{1c}) and T2D.

In ***Paper III*****,** the primary outcome was the dynamic and immediate changes in the plasma Pro-NT and plasma triglyceride levels and their correlations after the ingestion of cream and olive oil, respectively.

In ***Paper IV***, primary outcomes were ACM and CSM based on the International Classification of Diseases (ICD) codes Ninth or Tenth Revision (ICD-9; ICD-10) recorded as the underlying cause of death. During follow-up, the information of ACM and CSM were retrieved by linking the individual's 10-digit civil registration number with the Swedish National Cause of Death Register (SNCDR).(97) Mortality ascertainment was done according to registered underlying cause of death on the cause of death certificate in accordance with the ICD-9 or ICD-10 codes as follows:

Deaths due to CVD (ICD 9:390-459 or ICD 10: I 00-99), digestive tract diseases (ICD 9:520-579 or ICD 10: K 00-95), mental and behavioural disorders (ICD 9: 290-319 or ICD 10: F 01-99), symptoms and unspecific causes of death (ICD 9:780-

789 or ICD 10: R 00-99), respiratory diseases (ICD 9:460-519 or ICD 10: J 00-99), genito-urinary diseases (ICD 9: 580-629 or ICD 10: N 00-99), malignant cancers (ICD 9:140-239 or ICD 10: C 00-97), immunodeficiency and endocrine disorders (ICD 9:240-279 or ICD 10: D 80-89; E00-99), blood disorders (ICD 9:280-289 or ICD 10: D 50-D77), diseases in nerve system and ears (ICD 9:320-389 or ICD 10: G 00-99; H 00-99), infectious diseases (ICD 9: 001-139 or ICD 10: A 00-99; B 00-99), dermatological diseases (ICD 9: 680-709 or ICD 10: L 00-99), musculoskeletal diseases (ICD 9:710-739 or ICD 10: M 00-99), injuries and poison incidents (ICD 9:800-999 or ICD 10: U,V,W,X, Y 00-99). Subjects were followed until emigration, death, or end of follow-up (December 31, 2018).

3.4.2 Study Design

Cohort studies

By definition, a cohort is defined as "*any designed group of individuals who are followed or traced over a period of time*."(99) In epidemiology, a cohort study is the observational form of study design in which exposed and unexposed populations are compared without any interventions by the investigator. These studies can be prospective or retrospective in designs. In the *prospective study designs*, the groups are followed "longitudinally" over a period into the future and incidence of outcome are measured. Investigations are carried out before the outcomes of interest develop and information about the exposures are collected as they occur during the study. On the other hand, in *retrospective studies* some individuals of the study populations have already developed the outcome of interest and information about the exposures are collected from the past records. Association between outcomes and exposures are measured by analytical methods with confidence intervals (CI) which indicates the strength, direction, and range of an effect along with the likelihood of occurrence of a result by chance.

For ***Paper I and IV***, a prospective study design was used**.**

Cross-sectional studies

In the cross-sectional study design, exposures and outcomes are measured at the same time. This type of study design can measure prevalence of disease in the study population but does not provide information about causality and incidence of the disease. It is difficult to ascertain the time sequence of events in cross-sectional studies, and therefore they cannot determine cause and effect of the outcome. However, we can find correlations between exposures and outcomes at a particular point of time. These studies are conducted either before planning a cohort study or as a baseline for a cohort study and provides descriptive analyses for hypothesis generation in the investigation of many exposures and outcome variables.

For ***Paper II***, cross-sectional data from the MEDIM study was used.

3.4.3 Statistical Analysis

All data were analysed with IBM SPSS statistical software (version 24.0; IBM Corp., Armonk, NY). Two-sided p-values of <0.05 were regarded as statistically significant. The distribution of Plasma Pro-NT concentration was skewed to the right and therefore transformed using the natural logarithm.

Baseline measurements

Standard descriptive statistical analysis was used to summarize the baseline characteristics for the study cohorts.

The "*t-test*" is a test of significance. It gives an estimate, confidence interval and P-value, if the data are normally distributed in both groups and standard deviations are the same.

The "*Pearson Chi-square test*" is a non-parametric tool to analyse group differences when dependent variables are nominal. Chi-square test was used to assess differences in categorical variables in ***Paper I*** and ***Paper IV***.

The analysis of variance test (ANOVA) is a parametric method to compare the means between more than two groups where the dependent variable is continuous and normally distributed. "*P-values*" are obtained that indicates that at least one pair is significantly different from the other as used in ***Paper I***. Additionally for ***Paper II***, basic characteristics were compared between the Iraqi and Swedish-born group. To test the differences between these groups, analysis of covariance (ANCOVA) was used for continuous variables.

In ***Paper IV***, skewed and logarithmically transformed Pro-NT values were divided into population quartiles. Continuous variables of clinical characteristics were compared across quartiles by using ANOVA "linear trend" p-values.

Linear Regression

Linear regression measures the linear relationship between a continuous dependent and one or more independent variables. The assumptions for linear regression are:

- Linear relationship between X and Y.
- The observations are independent of each other.
- Homoscedasticity, i.e., residual values are normally distributed and have constant variance at every level of X.

In ***Paper I***, Linear regression was performed to measure the associations between Pro-NT (continuous dependent variable) and age, gender, BMI, HDL cholesterol, LDL cholesterol, systolic blood pressure, antihypertensive therapy, current smoking, and T2D being the explanatory, independent (predictor) variables. Changes in the Pro-NT (dependent variable) for one unit change in the predictors variables were estimated by the regression coefficient (β), keeping the effect of other variables constant in a multivariable linear regression. A *correlation* measures an association between the variables and a *correlation coefficient* "*r*" describes the strength of the linear correlation between the two variables. Correlation coefficient "r" can be between -1 and 1 and r=0 means no correlation between variables.

In ***Paper II***, multivariate linear regression analysis was used to study separate associations between "Pro-NT and insulin sensitivity index", "Pro-NT and disposition index", and "Pro-NT and HbA1c", respectively. Data are expressed as β-coefficients with 95% confidence intervals, CI.

In ***Paper III****,* linear regression models were used to assess the relationship between Pro-NT, TG, and p-glucose at fasting (0 hour) and then every hour for four hours. Changes at the maximal post-lipid ingestion peak concentration of Pro-NT, TG and glucose are compared with the baseline concentrations.

Survival analysis

Survival analysis or methods are used for "time to event" analysis. All individuals contribute to the *total time at risk* from inclusion until the *event of interest*. Even though, the individuals may not experience the *event of interest* itself, they provide information which is used for the estimation of the probability of the *event*. One of the important aspects of survival analysis is censoring. According to right censoring, individuals exit the study before the event of interest occurs or the study ends before the event.

Survival analysis includes, Kaplan-Meier test (non-parametric), log-rank test and Cox regression (semi-parametric).

A *Kaplan- Meier curve* is the graphical presentation of the probability to survive during a given length of time, while considering time in many small intervals.(100)

Cox regression model is a predictive model for the time to event data. It provides a survival function to predict the probability that an event of interest has occurred at a given time *t*, for given *predictor variables.* In all our Cox regression analysis, time to follow up has been used as time scale for calculating time to event. *Hazard ratios* are the regression coefficients obtained from Cox regression for the exposure variables. It is a risk ratio to estimate the relative survival or failure in one group when compared to the other group (reference group).

Cox model assumes that the effect of an exposure is constant over time known as the *proportional hazard assumption*. This assumption should be tested in Cox regression for analysis by graphical method, goodness of fit test and by using the time- dependent covariate terms in the analysis.(101)

The survival analysis including Kaplan-Meier curve and Cox-regression models are used in ***Paper I*** **and** ***Paper IV*** and proportional hazard assumptions were tested for Cox regression analysis.

Logistic Regression

This analytical method is most applicable when the dependent variable is categorical. When the outcome is binary, then binomial logistic regression is used. If the model is to be used for the prediction of a nominal dependent variable at two or more than two levels with one or more independent variable, then a multinominal logistic regression model is used. In this case, one level of the dependent variable is considered as the *reference category* and parameter estimates are relative to the reference group. Logistic regression is used to estimate the OR. *OR is the prediction of the fold change in risk due to the selected factors regarding the study outcome and they are estimated based on the independent variable*. The interpretation of multinomial logit is that for each unit change in the independent variable, the logit of being in a particular category relative to the reference group (outcome variable), is expected to change by its respective parameter estimate. The main assumption is that other variables are held constant.

In ***Paper II***, logistic regression was used for continuous and categorical variables respectively. All skewed variables (Pro-NT, insulin sensitivity index, ISI, and disposition index, DI) were $\log_{10}$-transformed before the analysis to approximate the normal distribution. Differences in the proportions between the groups were derived by logistic regression models adjusted for age and sex. Associations betweenT2D and Pro-NT with data adjusted for anthropometrics and life-style related risk factors were expressed as OR with 95% confidence intervals (CI) in 6 different models. (Table 9). In these models (Table 8 and Table 9), continuous independent variables were standardized to one unit variance (per 1 standard deviation, SD), in the strata of ethnicity and sex (z-scores). Correlation coefficients were assessed using Spearman´s test.

Associations between T2D and tertiles of Pro-NT with data adjusted for anthropometrics and lifestyle-related factors are expressed as OR with 95% CI in Table 3.

Interaction terms between tertiles of Pro-NT and country of birth and between Pro-NT and gender were assessed by logistic regression presenting OR. (Tables 8, 9 and 10)

Pearson Correlation analysis

In ***Paper III***, the relationship between post-lipid ingestion areas under the Curve (AUC) of Pro-NT and TG were studied following an oral lipid load of cream or olive oil. Changes at the maximal post-lipid ingestion concentration of Pro-NT and triglycerides versus the baseline concentrations were calculated every hour in 4 hours to calculate post-lipid ingestion AUCs of Pro-NT and triglycerides and then correlated by using Pearson correlation analysis.

4 Results

Paper I

Table 4. Baseline Characteristics of subjects free from CVD or T2D (n=4804).

Clinical Variable	Without CVD at baseline	Without T2D at baseline
Number	4804	4511
Age, years	69.1 ± 6.2	69.1 ± 6.2
Sex, men %, n	69.8%, n=3353	68%, n=3067
BMI, kg/m²	27.1 ± 4.2	26.8 ± 4.2
SBP, mm Hg	146 ± 20.4	144.8 ± 20.4
Current smoking, n (%)	951 (19.8%)	902 (20%)
HDL cholesterol, mmol/L	1.38 ± 0.4	1.40 ± 0.4
LDL cholesterol, mmol/L	3.7 ± 1.0	3.7 ± 1.0
Glucose, mmol/L	5.9 ± 1.5	5.5 ± 1.5
Antihypertensive treatment, n (%)	1685 (35%)	1578 (35%)
Type 2 diabetes, n (%)	504 (10.5%)	-
Fasting proneurotensin, pmol/L	117 ± 111	117 ± 111

The main findings of ***Paper I*** were that high fasting Pro-NT concentrations independently predicted the development of CVD in the elderly population (mean age~70 years) and the development of incident T2D, but only in women.

At baseline, 4804 subjects free from CVD and 4511 subjects free from T2D (mean age of 69.1 ± 6.2) with complete data on covariates were studied. During a mean follow-up period of 5.4 ± 1.6 years for the analysis of incident CVD (n=4804), 456 cases of incident CVD (110 women, 346 males) were reported. In the analysis of incident T2D (n= 4511), 222 cases (71 women, 151 men) occurred during the follow up time of 5.5 ± 1.5 years.

Age and fasting plasma Pro-NT were weakly but significantly inversely associated in this elderly population in both genders. In men, each year increase in age was associated with 0.037 [β 95% confidence interval] [(0.032 – 0.042) $P<0.001$] lower values of log-transformed Pro-NT concentrations. Correspondingly, in women, each

year increase in age was associated with 0.024 [β 95% confidence interval] [(0.013 – 0.036) *P<0.001*] lower values of log-transformed Pro-NT concentrations. After adjusting for age differences in both genders, no significant associations were observed between any of the risk factors and the baseline characteristics listed in (Table 4).

In the Kaplan-Meier survival analysis, the cumulative risk of first CVD event was significantly increased in the individuals with above vs below median levels of Pro-NT (P= 0.045). Following stratification for sex, significant associations were seen in women (P= 0.044), but not in men (*P=0.7*). Effects of traditional risk factors on the associations were assessed by additional adjustments for classical risk factors, but associations remained significant in the entire population. (Table 5). Each SD increment of Pro-NT increased the risk of CVD by 10% [HR 1.10 (95%CI 1.01-1.21) *P=0.037*] and subjects in above vs below the median level of Pro-NT had 27 % increased risk of incident CVD in both genders [HR 1.27 (95%CI 1.06-1.54*) P= 0.011*]. (Table 5) There was no significant interaction between Pro-NT and gender on the outcome of incident CVD.

The risk of developing T2D in the individuals with above vs below median levels of Pro-NT was not significant (*P= 0.11*) during the follow-up time, in crude Kaplan-Meier analysis. This remained insignificant even after adjustment for T2D -related risk factors in the overall population. However, a gender-stratified analysis showed significant associations between above vs below the median Pro-NT and incidence of T2D in women (*P= 0.004*), but not in men (*P=1.000*) in crude Kaplan-Meier analysis. (Figure 8)

In fully adjusted models after gender stratification, each SD increment of fasting Pro-NT increased the risk of T2D in women with 28% [HR 1.28 (95%CI 1.30-1.59) *P=0.025*]. Women above vs below the median level of Pro-NT had 41% increased risk of incident T2D [HR 1.41 (95%CI 1.10-1.80) *P= 0.007*]. No significant association between Pro-NT and incident T2D was observed in fully adjusted models. (Table 6)

Table 5. Pro-NT in the prediction of incident cardiovascular disease (CVD) events in all participants, women and men.

ALL SUBJECTS				
	Per 1SD increment N=4804 (456 events)	**P-value**	**Above vs below median Pro-NT(88.2 pmol/L)**	**P-value**
HR (95% CI)	1.10 (1.01-1.21)	*0.037*	1.27 (1.06-1.54)	*0.011*
WOMEN				
	Per 1SD increment N=1533 (110 events)	**P-value**	**Above vs below median of Pro-NT (78.6 pmol/L)**	**P-value**
HR (95% CI)[c]	1.14 (0.96-1.37)	*0.146*	1.41 (0.97-2.07)	*0.073*
MEN				
	Per 1SD increment N=3271 (346 events)	**P-value**	**Above vs below median of Pro-NT (92.0 pmol/L)**	**P-value**
HR (95% CI)[c]	1.09 (0.98-1.21)	*0.106*	1.22 (0.99-1.51)	*0.069*

Abbreviations: CI, confidence interval; HR, Hazard ratio

Table 6: Pro-NT in the prediction of incident T2D events in all subjects, women and men.

ALL SUBJECTS				
	Per 1SD increment N=4511 (222events)	**P-value**	**Above vs below median of Pro-NT (88.2 pmol/L)**	**P-value**
HR (95% CI)	1.05 (0.91-1.20)	*0.521*	1.09 (0.95-1.25)	*0.225*
WOMEN				
	Per 1SD increment N=1433 (71events)	**P-value**	**Above vs below median of Pro-NT (78.6 pmol/L)**	**P-value**
HR (95% CI)	1.28(1.30-1.59)	*0.025*	1.41 (1.10- 1.80)	*0.007*
MEN				
	Per 1SD increment N=3078 (151 events)	**P-value**	**Above vs below median of Pro-NT (92.0 pmol/L)**	**P-value**
HR (95% CI)	0.95 (0.80 – 1.12)	*0.524*	0.95 (0.81-1.12)	*0.568*

Abbreviations: CI, confidence interval; HR, Hazard ratio

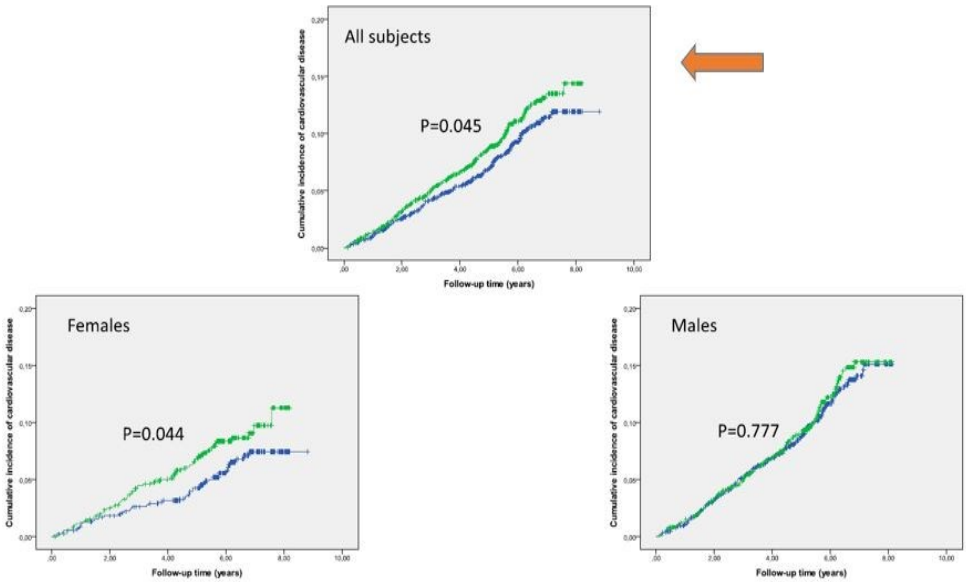

FIGURE 7. Kaplan Meier plots showing the cumulative incidence of cardiovascular disease in all subjects, women and men. Arrow indicates significant result in all subjects.

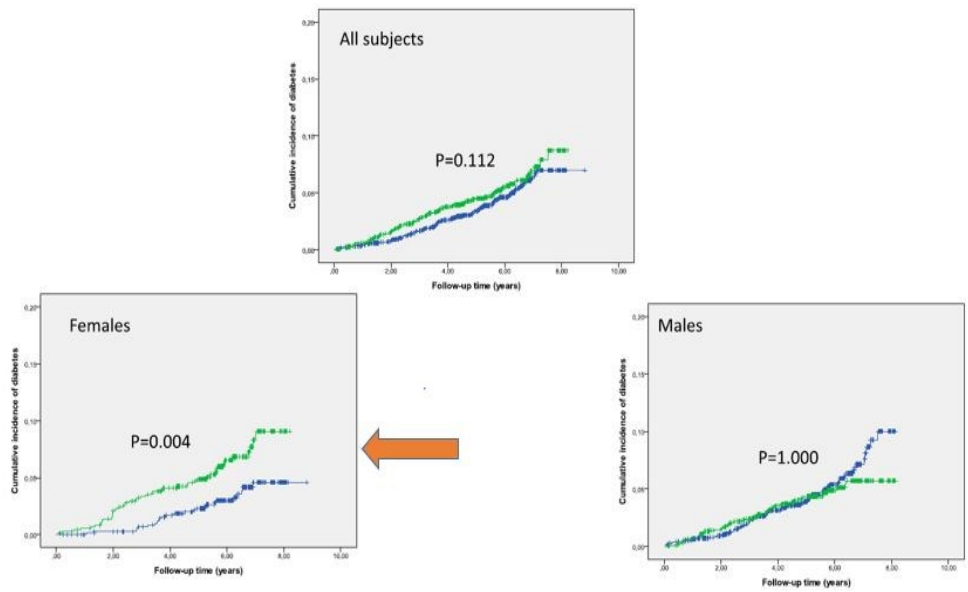

FIGURE 8: Kaplan Meier plots showing cumulative incidence of T2D in all subjects, women, and men. Arrow indicates significant result in women.

Paper II

Table 7. Characteristics of participants in the MEDIM study, born in Iraq or in Sweden. Data are presented as means (standard deviations, SD), numbers (%) or medians (inter-quartile range, IQR).

	Born in Iraq N = 1398	Born in Sweden N= 757	*p - value*
Age, years	46.2 (9.6)	49.5 (11.2)	*<0.001*
Male sex, *n* (%)	819 (58.6)	378 (49.9)	*0.010*
Proneurotensin, pmol/L[a]	137.5 (111.1–164.7)	119.8 (99.9–145.3)	*<0.001*
Body mass index, kg/m²	29.3 (4.5)	27.3 (4.7)	*<0.001*
Waist circumference in men, cm	99.3 (10.6)	93.1 (10.9)	*0.002*
Waist circumference in women, cm	97.8 (11.7)	89.2 (14.1)	*<0.001*
Systolic Blood Pressure, mmHg[b]	127 (15.6)	134 (19.1)	*<0.001*
Diastolic Blood Pressure, mmHg[b]	77 (9.8)	80 (11.4)	*<0.001*
HbA_{1c} mmol/mol	37.9 (9.9)	36.3 (8.1)	*<0.001*
HbA_{1c} %	4.6 (0.9)	4.5 (0.8)	*<0.001*
Plasma Cholesterol LDL, mmol/L[c]	3.2 (0.8)	3.4 (0.9)	*<0.001*
Plasma Triglycerides, mmol/L[c]	1.6 (1.0)	1.2 (0.8)	*<0.001*
Insulin Sensitivity Index[a]	76.9 (58.4–101.1)	102.3 (77.7–133.0)	*<0.001*
Corrected Insulin Response[a,d]	167.8 (116.0 – 237.4)	140.1 (100.1–196.7)	*<0.001*
Disposition Index[a,d]	12413.3 (8316.3 – 18692.7)	13805.9 (9560.7 – 20150.4)	*0.028*
Type 2 diabetes, %	162 (11.6)	44 (5.8)	*<0.001*
Cardiovascular Disease, %	54 (3.9)	41 (5.4)	*0.058*
Family History of type 2 diabetes, %	716 (51.2)	200 (26.4)	*<0.001*

a Presented as median (inter-quartile range, IQR).

b Including participants without antihypertensive drug medication

c Including participants without lipid lowering drug medication

d Only included cases where the glucose level at 30 min was >4.44 mmol/l and higher than the fasting glucose level [21]

In total, 1398 subjects (819 men, 579 women) born in Iraq, aged 46.2 ± 9.6 years, and 757 subjects (400 men, 357 women) born in Sweden, aged 49.5 ±11.2 years, participated in this cross-sectional study. In age- and sex-adjusted analysis of clinical and biomedical characteristics of study subjects at baseline, fasting Pro-NT levels and BMI were significantly higher amongst Iraqi-born as compared to Swedish-born subjects. Higher BMI was positively associated with higher Pro-NT levels (β 0.004; 95%CI: 0.001-0.006; *P=0.016*). Similarly, Iraqi origin remained positively associated with Pro-NT (β 0.123; 95%CI: 0.082-0.163; *P<0.001)* even after adjusting for age, sex, and BMI. Insulin sensitivity index (ISI), corrected

insulin response (CIR) and insulin disposition index (Dio) were lower at baseline in the Iraqi-born subjects as compared to Swedish-born subjects. Prevalence of T2D and a positive family history of T2D was higher in Iraqi-born subjects as compared to Swedish-born subjects as observed in studies previously.(78) However, there were no significant differences in the prevalence of CVD in our study population.

Pro-NT and risk of T2D

Associations between T2 D and Pro-NT were studied by binary logistic regression analysis. Results showed that the odds of T2D increased approximately by 1.5-fold per 1 unit increase (1 SD) of Pro-NT in both Iraqi- and Swedish-born participants (Table 8). These associations remained significant in the fully adjusted model for age, sex, country of birth, BMI, and fatty liver index in six different statistical models, Model I to Model VI. (Details in the manuscript).

Further, Iraqi-born individuals within the third tertile displayed almost five times higher odds of T2D as compared to the reference group of native Swedes within the first tertile of Pro-NT (Table 10). For each tertile, higher odds of T2D in Iraqis as compared to native Swedes were observed. However, this model did not have the statistical power to detect any significant interactions between country of birth and Pro-NT, or between gender and Pro-NT.

Stepwise, multivariable linear regression analysis between insulin action (ISI), insulin secretion (DIo) and HbA1c as dependent variables and Pro-NT as independent variable showed that Iraqi origin modified the relationship of Pro-NT with the glucose regulation indices. Significant interactions between country of birth and Pro-NT were observed in all three models of indices of glucose regulations. ($P_{interaction\ ISI}$ = 0.048; $P_{interaction\ DI}$=0.014 and $P_{interaction\ HbA1c}$=0.029). There were no significant interactions observed between Pro-NT and gender.

In the Scatter plot diagrams in Figures 9-11, regression lines represent the relationship between glucose regulation assessed as insulin action (ISI), insulin secretion (DIo) and HbA1c, respectively, with log-transformed Pro-NT in the Iraqi-born and Swedish-born populations. In the Iraqi-born population only, insulin action (ISI) and insulin secretion (DIo) showed significant inverse (negative) correlations with Pro-NT ($P_{ISI}<0.001$; $P_{DIo}=0.015$) showing that insulin secretion and action decreases with an increase in Pro-NT as shown in Figures 9 and 10, respectively. HbA1c showed a significant positive correlation with Pro-NT ($P_{HbA1c}=0.036$), indicating that HbA1c increases with increasing Pro-NT levels. (Figure 11)

Table 8. Associations between insulin action (insulin sensitivity index), insulin secretion (disposition index) and HbA1c as dependent variables with Pro-NT as the independent variable.

Proneurotensin[a]								
Dependent variable		**Total study population**		**Born in Iraq**		**Born in Sweden**		$P_{Interaction}$
								ProNT[a] *Country of Birth
	Model	b	**95% CI**	**b**	**95% CI**	**B**	**95% CI**	
Insulin Sensitivity Index [a]								
	Model I	-.019	**-.032 to -.007**	**-.029**	**-.044 to -.014**	**-.005**	**-.025 to .015**	
	Model II	-.020	**-.032 to -.008**					*0.057*
	Model III	-.017	**-.028 to -.007**	**-.026**	**-.040 to -.013**	-.004	-.021 to .012	
	Model IV	-.018	**-.029 to -.007**					*0.050*
	Model V	-.023	**-.033 to -.014**	**-.031**	**-.043 to -.018**	-.010	-.026 to .006	
	Model VI	-.023	**-.033 to -.013**					***0.048***
Disposition Index [a,b]								
	Model I	-.014	-.033 to .005	**-.033**	**-.057 to -.010**	.019	-.011 to .049	
	Model II	-.014	-.033 to .004					***0.008***
	Model III	-.013	-.031 to .005	**-.032**	**-.056 to -.009**	.020	-.009 to .050	
	Model IV	-.013	-.032 to .005					***0.007***
	Model V	-.015	-.033 to .004	**-.033**	**-.057 to -.009**	.018	-.013 to .048	
	Model VI	-.015	-.033 to .004					***0.014***
HbA_{1c}								
	Model I	.749	**.038 to 1.120**	**1.08**	**0.06 to 1.59**	.112	-.366 to .589	
	Model II	.746	**.377 to 1.114**					***0.014***
	Model III	.729	**.362 to 1.096**	**1.07**	**.572 to 1.574**	.071	-.397 to .539	
	Model IV	.723	**.359 to 1.088**					***0.010***
	Model V	.779	**.411 to 1.146**	**1.07**	**.569 to 1.580**	.194	-.274 to .662	
	Model VI	.772	**.405 to 1.139**					***0.029***

Data was adjusted for anthropometrics and country of birth, Model I to Model VI. (Details of models in manuscript)

a base 10 logarithm

b CIR and DIo only included cases where the glucose level at 30 min was >4.44 mmol/l and was greater than the fasting glucose level [21].

c Regression coefficients (β and odds ratios respectively) for continuous independent variables in Model I to VI were standardized to a unit variance (per 1 standard deviation) in the strata of ethnicity and sex (z-scores).

Table 9. Associations between T2D and Pro-NT with data adjusted for anthropometrics and lifestyle-related risk factors, Model I to Model VI.(Details of models in manuscript)

Proneurotensin[a]								
		Total study population		Born in Iraq		Born in Sweden		$P_{Interaction}$ ProNT[a] *Country of Birth
		OR	95% CI	OR	95% CI	OR	95% CI	
Dependent variable	Model							
T2D								
	Model I	1 481	1.253 to 1.750	1 470	**1.218 to 1.773**	**1 541**	**1.054 to 2.252**	
	Model II	1 483	1.254 to 1.755					NS
	Model III	1 523	1.298 to 1.788	1 453	**1.204 to 1.755**	**1 535**	**1.047 to 2.251**	
	Model IV	1 470	1.241 to 1.741					NS
	Model V	1 534	1.282 to 1.835	1 484	**1.213 to 1.817**	**1 713**	**1.141 to 2.572**	
	Model VI	1 531	1.278 to 1.835					NS

Table 10. Prevalent T2D and its associations with country of birth and tertiles of Pro-NT assessed using logistic regression and expressed as odds ratios (OR) with 95% confidence intervals (CI's).

	Total study population		
		OR	**95% CI**
Age, years per 1 SD		**2.44**	**2.05 2.90**
Male sex		**1.51**	**1.06 2.16**
BMI kg/m², per 1 SD		**1.52**	**1.29 1.80**
Family History of type 2 diabetes		**1.90**	**1.33 2.71**
Proneurotensin			
- **First tertile**	Born in Sweden	Reference	
	Born in Iraq	**2.02**	**0.82 4.97**
- **Second tertile**	Born in Sweden	0.61	0.17 2.15
-	Born in Iraq	**3.57**	**1.53 8.37**
- **Third tertile**	Born in Sweden	**3.49**	**1.41 8.64**
-	Born in Iraq	**4.82**	**2.13 10.89**

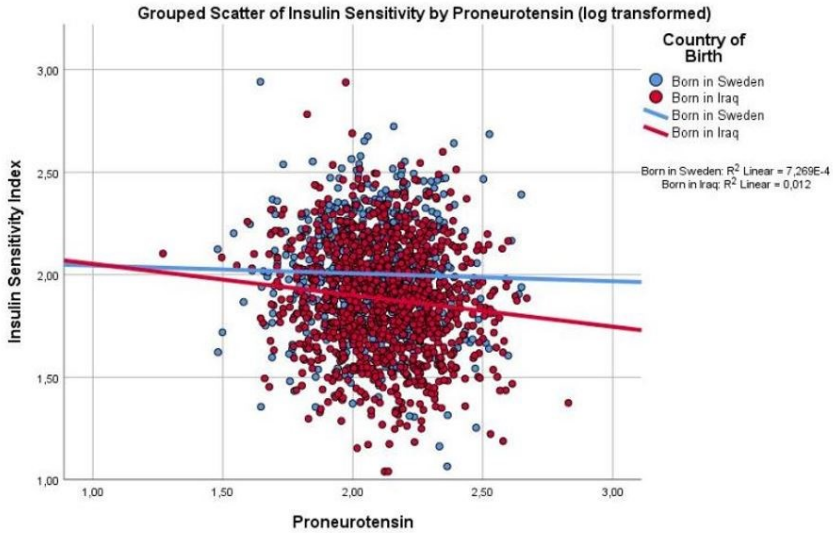

Figure 9: Association between insulin action (insulin sensitivity index, ISI) as dependent variable and Pro-NT as the independent variable in regression model adjusted for anthropometrics and country of birth. (In the Iraqi-born population *P<0.001)*.

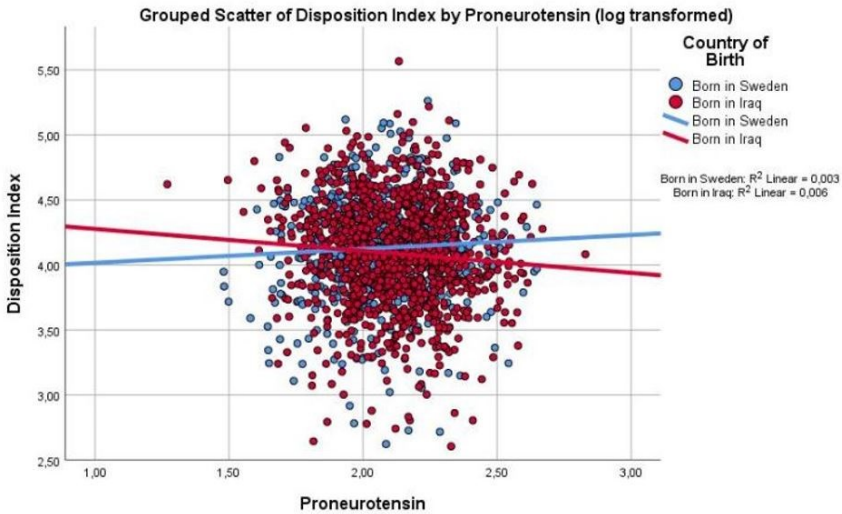

Figure 10: Association between Disposition Index (DIo) as dependent variable and Pro-NT as the independent variable in regression model adjusted for anthropometrics and country of birth. (In the Iraqi-born population *P=0.015)*

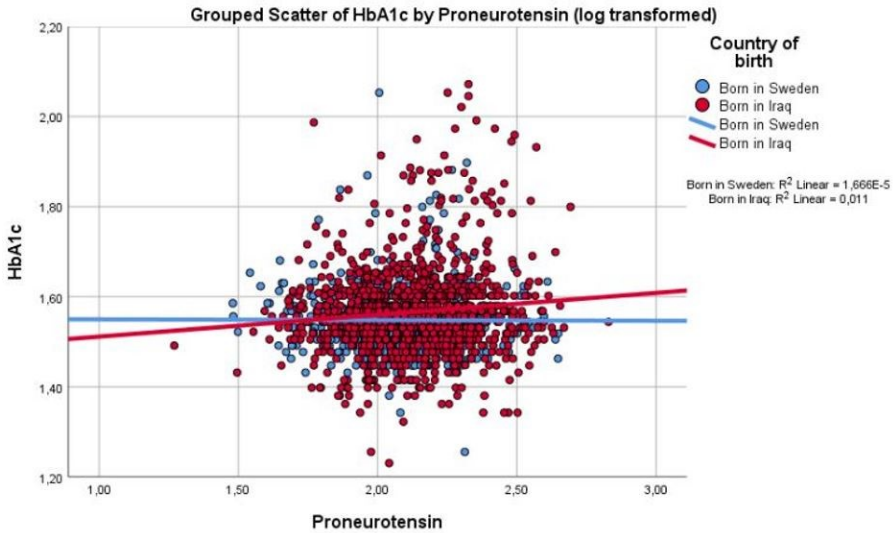

Figure 11: Association between HbA1c as dependent variable and Pro-NT as the independent variable in regression model adjusted for anthropometrics and country of birth. (In the Iraqi-born population *P=0.036)*

Paper III

Table 11: Anthropometric and blood parameters of study participants.

Parameter	Mean ± SD
N (men/women)	22 (10/12)
Age(years)	31 ± 9.5
Pro-NT[mmol/L]	122 ± 48
SBP (mmHg)	115 ± 16
DBP (mmHg)	66 ± 9
Cholesterol [mmol/L]	4.7 ± 0.97
HDL Cholesterol [mmol/L]	1.7 ± 0.28
LDL Cholesterol [mmol/L]	2.9 ± 0.82
Insulin[mmol/L]	7.1 ± 4.0
Glucose [mmol/L]	5.2 ± 0.53
Triglycerides [mmol/L]	0.71 ± 0.25

A total of 22 healthy subjects (10 men, 12 women) aged 31±9.5 years were examined to observe the dynamic changes in plasma triglycerides, glucose, and plasma Pro-NT levels after ingestion of equimolar amount of cream (50g fat) or olive oil (50g fat), respectively. Anthropometric and blood parameters of study subjects are shown in Table 11.

Results after cream ingestion

Plasma Pro-NT started to rise significantly after1 hour following cream ingestion. It reached its maximum level of mean 22 (12-31) pmol/L ($P< 0.001$) after 2 hours and then started to decline after that. Accordingly, triglycerides also started to rise significantly after 1 hour and reached its maximum level of mean 0.60 (0.43-0.78) mmol/L ($P< 0.001$) after 3 hours, but after that it started to decline. The area under the curve (AUC) for Pro-NT was 49 ± 74 pmol/L x h and AUC for triglycerides was 1.5 ± 0.95 mmol/L x h, respectively. (Figure 12)

Results after olive oil ingestion

Plasma Pro-NT started to rise significantly after 1 hour following olive oil ingestion and reached its maximum level of mean 62 (46-78) pmol/L ($P< 0.001$) after 3 hours and started to decline after that. Accordingly, triglycerides also started to rise significantly after 2 hours and reached its maximum level of mean 0.32 (0.18-0.45) mmol/L ($P< 0.001$) after 3 hours. It started to decline after that. The area under the curve (AUC) for Pro-NT was 196 ± 115 pmol/L x h and AUC for triglycerides was 0.71 ± 0.77 mmol/L x h, respectively. (Figure 13)

Significant correlations of post-lipid load AUC for Pro-NT and plasma triglycerides were observed both after ingestion of cream (r=0.49, *P=0.021*) and after ingestion of olive oil (r=0.55, *P=0.008*) showing the pivotal role of Pro-NT in intestinal fat absorption. (Figures 12, 13)

In the study of *Pro-NT and plasma glucose concentrations* after lipid ingestion of cream or olive oil, plasma glucose levels significantly decreased at all observations points as compared to the fasting plasma glucose. The AUC of glucose was not significantly correlated to the AUC of Pro-NT. (Table 13)

Table 12: Area Under the Curve (AUC) for Pro-NT and triglycerides after ingestion of cream or olive oil.

Fat load	Subjects	mean AUC standard deviation		P-value
Cream	22	AUC Pro-NT	49 ± 74	*<0.001*
		AUC Triglycerides	1.5 ± 0.95	*0.004*
Olive Oil	22	AUC Pro-NT	196 ± 115	*<0.001*
		AUC Triglycerides	0.71 ± 0.77	*0.004*

Table 13: Changes in plasma glucose concentrations at all-time points, both after cream and after olive oil ingestion.

Time (hour)	Cream			Olive Oil		
Ref. 0 hour	Δ Glucose Mean	95% Confidence Interval	P-value	Δ Glucose Mean	95% Confidence Interval	P-value
1 hour	- 0.54	(- 0.75 - - 0.32)	*<0.001*	- 0.39	(- 0.58 - - 0.20)	*<0.001*
2 hour	- 0.33	(- 0.54 - - 0.11)	*0.005*	- 0.42	(- 0.64 - - 0.20)	*0.001*
3 hour	- 0.30	(- 0.46 - - 0.13)	*0.002*	- 0.31	(- 0.51 - - 0.11)	*0.004*
4 hour	- 0.28	(- 0.49 - - 0.07)	*0.011*	- 0.29	(- 0.46 - - 0.11)	*0.003*

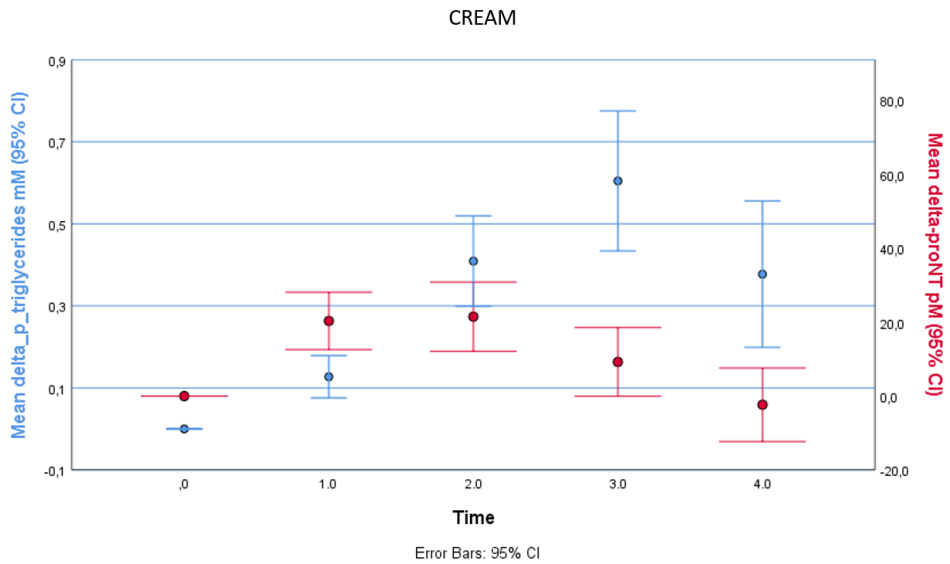

Figure 12: Changes of Pro-NT and triglycerides after cream and correlation of post-lipid load Area Under Curve (AUC) for Pro-NT with the AUC for triglycerides after ingestion of cream.

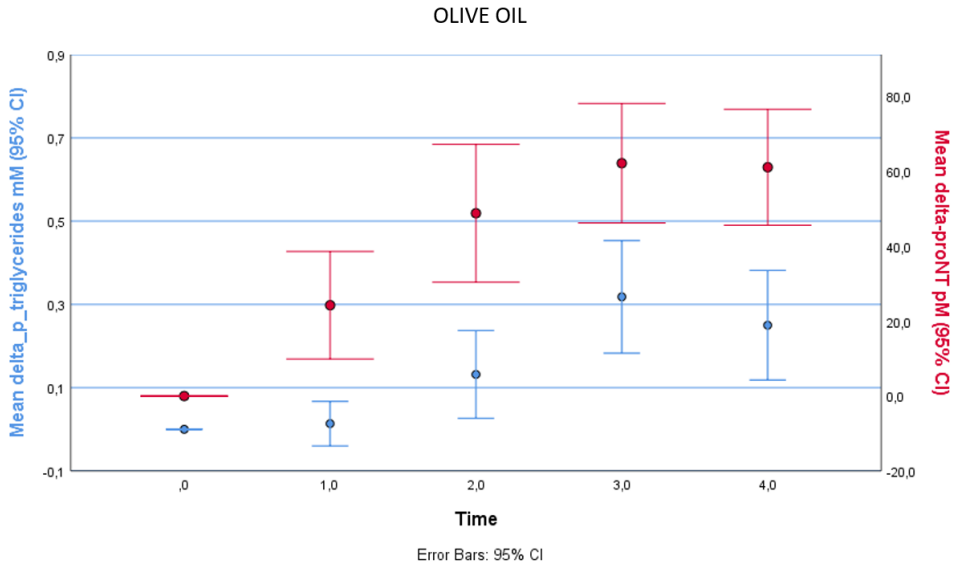

Figure 13: Changes of Pro-NT and triglycerides after olive oil and correlation of post-lipid load Area Under Curve (AUC) for Pro-NT with the AUC for triglycerides after ingestion of olive oil.

Paper IV

Table 14: Baseline characteristics of the study participants stratified by quartiles (Q1-Q4) of Pro-NT.

	Pro-NT	Pro-NT	Pro-NT	Pro-NT	*P* for trend
	Q1	Q2	Q3	Q4	
No. of participants	1158	1158	1158	1158	
Men, n (%)	558 (48.2%)	497 (42.9%)	447 (38.6%)	467 (40.3%)	*<0.001*
Age, mean (SD), years	58.0 ± 6.00	57.7 ± 5.98	57.6 ± 5.96	57.7 ± 5.97	*0.309*
SBP, mean (SD), mmHg	142.8 ± 19.7	141.6 ± 18.6	142.8 ± 19.7	142.2 ± 18.8	*0.430*
Antihypertensive therapy, No. (%)	188 (16.2%)	197 (17%)	180 (15.5%)	224 (19.3%)	*0.112*
Type 2 diabetes, No. (%)	77 (6.7%)	100 (8.7%)	100 (8.7%)	141 (12.3%)	*<0.001*
BMI, mean (SD), kg/m²	25.8 ± 3.84	25.7 ± 3.75	25.9 ± 4.01	26.0 ± 4.12	*0.071*
LDL-C mean (SD), mmol/L	4.16 ± 1.02	4.16 ± 0.96	4.21 ± 0.98	4.13 ± 0.98	*0.741*
HDL-C mean (SD), mmol/L	1.36 ± 0.36	1.39 ± 0.38	1.39 ± 0.37	1.39 ± 0.38	*0.018*
Current smokers, n (%)	298 (25.7%)	278 (24.0%)	290 (25.0%)	354 (30.6%)	*0.007*

Abbreviations: BMI (body mass index), LDL-C (Low-density lipoprotein cholesterol), HDL-C (High-density lipoprotein cholesterol), SBP (systolic blood pressure), Pro-NT (Proneurotensin).

Of 4632 patients (42.5% men, 57.5% women), 1906 (31.3%) died during a follow-up period of 20±3 years. Median age of participants at baseline was 57.7 years (interquartile range 51.7-63.7 years).

The clinical characteristics of the study participants were stratified by quartiles of Pro-NT. It was observed that Pro-NT in higher quartiles was significantly related to prevalence of T2D (*P< 0.001*), current smoking (*P=0.007*), HDL- cholesterol and female gender in the crude baseline analysis. (Table 14)

Pro-NT and all-cause mortality

In the Cox proportional hazard model with Pro-NT as a continuous variable, adjusted for age and sex, fasting Pro-NT was associated with an increased mortality risk in quartile **(Q) 4 vs. Q 1 analysis** with HR 1.33, (95% CI,1.17-1.42; *P<0.001*). No specific interactions were observed between Pro-NT and gender stratification on mortality.

Similarly, in **Q4 vs Q1-3 analysis**, having Pro-NT ≥ 149 pmol/L, increased the relative risk of ACM, HR 1.29 (95% CI, 1.17-1.43; *P<0.001*) in a model adjusted for age and sex, and 1.20 (95% CI, 1.08-1.33; *P<0.001*) after additional adjustment for cardiovascular risk factors. Mortality risk in quartiles 2 and 3 respectively, did not differ from ACM in quartile 1. (Table 15)

A Kaplan-Meier plot for cumulative proportion of ACM by Pro-NT quartiles during the follow-up shows that Q 4 of Pro-NT deviates from the three lower quartiles. (Fig. 14)

Pro-NT and cause-specific mortality

For a total of 1906 deaths, significant association of Pro-NT in **Q 4 vs Q 1-3** and CSM in the Cox-regression model were observed, when adjusted for age, sex, BMI, LDL-C, HDL-C, T2D, smoking, systolic blood pressure and antihypertensive drug treatment. Around 595 deaths (31.2%) were reported due to CVD, HR of 1.41 (95% CI, 1.18 -1.68; *P<0.001*), 42 deaths (2.20%) due to digestive tract diseases with a HR 2.53 (95% CI, 1.37- 4.67; *P=0.003*), 90 deaths (4.72%) due to mental and behavioral diseases with a HR 1.62 (95% CI, 1.04 - 2.52; *P=0.032*), and 64 deaths (3.36%) were due to unspecific causes with a HR of 1.91 (95% CI, 1.15 - 3.19; *P=0.013*). There was no significant relationship between Pro-NT levels and deaths due to cancer, infections, neurological or other causes as listed in Table 16.

In the multivariate Cox regression analysis with Pro-NT as a continuous variable in relation to cause-specific survival, Pro-NT remained a positive predictor of deaths due to CVD and GIT diseases. However, the results were not significant for deaths due to mental diseases or unspecific diseases after adjustment for age, sex, body mass index, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, T2D, smoking, systolic blood pressure, and antihypertensive treatment. This is added in Supplementary Table S1 in the manuscript.

The association between Pro-NT and cancer deaths was non-significant with HR 1.12 (95% CI 0.93-1.95), *P=0.222* in an age- and sex-adjusted model, like the result of “all cancer mortality” as presented in Table 16.

Table 15: Event rates and multivariate adjusted Cox Proportional Hazards Models for baseline Pro-NT in relation to all-cause mortality.(Adjusted for age and sex).

All-cause mortality	**Q1**	**Q2**	**Q3**	**Q4**	***P* for trend**
All participants					
No./ events No.	1158/458	1158/442	1158/470	1158/536	
Pro-NT, median (range), pmol/L	60.0 (3.00 -75.8)	89.0 (75.9-105)	123 (105-149)	190.4 (149-1150)	
HR (95% CI)	REF (1.0)	0.99 (0.87-1-12)	1.10 (0.97-1.26)	1.33 (1.17-1.50)	*<0.001*
Women					
No./events No.	600/194	661/213	711/252	691/291	
Pro-NT, median, pmol/L	60.4 (3.00 -75.8)	89.1 (75.9-104.6)	122.6 (104.7-148.5)	192.0 (148.6-1154.5)	
HR (95% CI)	REF (1.0)	1.03 (0.85-1.25)	1.11 (0.92-1.34)	1.42 (1.19-1.70)	*<0.001*
Men					
No./events No.	558/264	497/229	447/218	467/245	
Pro-NT, median, pmol/L	59.9 (0.00-75.8)	89.6 ((75.9-104.5)	123.3 (104.8-148.3)	188.4 (148.6-1057.4)	
HR (95% CI)	REF (1.0)	0.95 (0.80-1.14)	1.10 (0.92-1.32)	1.24 (1.04-1.48)	*0.014*

Table 16: Event Rates and multivariate Cox Proportional Hazards Models for quartiles (Q1 – Q4) of baseline Pro-NT in relation to cause-specific mortality.

	Age and sex adjusted				**Fully adjusted***			
	All participants	**Q 1-3**	**Q 4**	***P value***	**All participants**	**Q 1-3**	**Q 4**	***P value***
All-cause mortality								
No./events No.	4632/1906	3474/1370	1158/536		4478/1823	3365/1316	1113/507	
HR (95% CI)		Ref 1.0	1.29 (1.17-1.43)	*<0.001*			1.20 (1.08-1.33)	*0.001*
Cardiovascular diseases								
No./events No.	4632/595	3474/419	1158/176		4478/562	3365/397	1113/165	
HR (95% CI)			1.41 (1.18-1.68)	*<0.001*			1.30 (1.08-1.56)	*0.005*
Gastrointestinal diseases								
No./events No.	4632/42	3474/24	1158/18		4478/42	3365/24	1113/18	
HR (95% CI)			2.53 (1.37-4.67)	*0.003*			2.37 (1.28-4.39)	*0.006*
Mental and behavioral diseases								
No./events No.	4632/90	3474/61	1158/29		4478/89	3365/61	1113/28	
HR (95% CI)			1.62 (1.04-2.52)	*0.032*			1.56 (0.99-2.34)	*0.057*
Unspecific diseases								
No./events No.	4632/64	3474/41	1158/23		4478/62	3365/40	1113/22	
HR (95% CI)			1.91 (1.15-3.19)	*0.013*			1.80 (1.07-3.04)	*0.028*
Cancer								
No./events No.	4632/683	3474/509	1158/174		4478/658	3365/493	1113/165	
HR (95% CI)			1.10 (0.92-1.30)	*0.293*				
Infectious diseases								
No./events No.	4632/41	3474/30	1158/11		4478/35	3365/25	1113/10	
HR (95% CI)			1.25 (0.62-2.50)	*0.531*				
Endocrine diseases								
No./events No.	4632/48	3474/34	1158/14		4478/45	3365/32	1113/13	
HR (95% CI)			1.39 (0.75-2.59)	*0.300*				

External causes								
No./events No.	4632/58	3474/41	1158/17		4478/52	3365/39	1113/13	
HR (95% CI)			1.35 (0.76-2.37)	*0.303*				
Musculoskeletal diseases								
No./events No.	4632/09	3474/6	1158/03		4478/09	3365/06	1113/03	
HR (95% CI)			1.63 (0.41-6.56)	*0.487*				
Urogenital diseases								
No./events No.	4632/25	3474/20	1158/05		4478/24	3365/19	1113/05	
HR (95% CI)			0.91 (0.34-2.43)	*0.854*				
Respiratory diseases								
No./events No.	4632/125	3474/92	1158/33		4478/122	3365/90	1113/32	
HR (95% CI)			1.22 (0.82-1.1)	*0.331*				
Neurological diseases								
No./events No.	4632/107	3474/80	1158/27		4478/104	3365/77	1113/27	
HR (95% CI)			1.11 (0.72-1.72)	*0.647*				
Hematological diseases								
No./events No.	4632/05	3474/04	1158/01		4478/05	3365/04	1113/01	
HR (95% CI)			0.88 (0.99-7.88)	*0.91*				

⁎ Adjusted for age, sex, BMI (body mass index), LDL-C (low-density lipoprotein cholesterol), HDL-C (high-density lipoprotein cholesterol), T2D, smoking, systolic blood pressure and antihypertensive drug treatment.

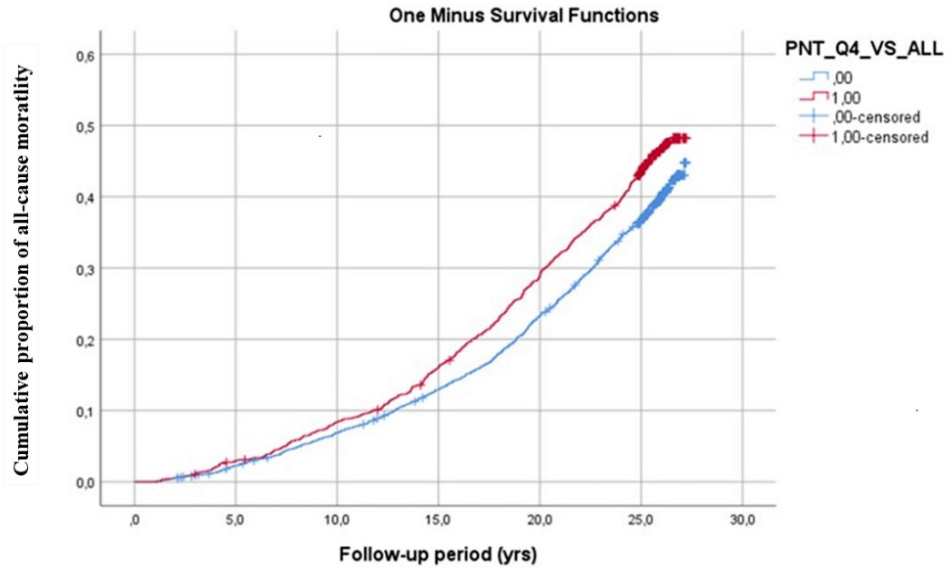

Figure 14: Kaplan-Meier plot showing the cumulative proportion of all-cause mortality during follow up in Q4 vs Q1-3 of baseline Pro-NT.

5 Discussion

In this thesis, we studied the association between fasting Pro-NT and cardiometabolic traits as well as risk prediction of fatal and non-fatal events.

5.1 Pro-NT and cardiometabolic risk prediction

Evidence of significant associations of fasting plasma *Pro-NT and risk of CVD* were previously explored in middle-aged Swedish and United States populations (102, 103). The first paper in this thesis (***Paper I***) reported that even in the elderly Swedish population of mean age of 70 years, plasma Pro-NT independently predicted incidence of CVD in both men and women. However, there was a lack of gender-specific associations between Pro-NT and CVD in the elderly population which is also reported in Framingham Heart study.(103) Similarly, previously reported positive associations between BMI and Pro-NT in the middle-aged Swedish and US populations (102, 103) were not observed in the current study. This emphasizes that determinants of obesity and CVD risk in relation to neurotensin are partially different in the elderly population as BMI and body fat distribution varies with age, sex, and ethnicity. However, independent of obesity-related CVD risk, there are several mechanisms that links the neurotensin system to CVD. In rodents, manipulation of NTSR3 has shown a novel role in atherosclerosis and vascular calcification. Similarly, in humans, genetic variation of NTSR3 increases the risk of coronary artery disease. (104, 105) It can be speculated that NTSR3 mediated mechanisms explain the neurotensin related increased CVD risk in elderly populations as atherosclerosis becomes more common with age.

In the study of *Pro-NT and incident* T2D *risk* in the same elderly population in ***Paper I***, fasting plasma Pro-NT independently predicted the development of T2D in women, but not in men. A possible explanation for the female-specific association between Pro-NT and T2D could be related to the influence of sex hormones (estrogen). Studies have shown that neurotensin regulating genes are dramatically upregulated and modulated by estrogen exposure.(106) Even though women in the current study were predominantly post-menopausal, the exposure of estrogen hormone on the neurotensin system in women during fertile years could possibly play an important role in the development of T2D in women as compared to men.

In ***Paper II***, fasting Pro-NT levels were significantly higher in Middle-Eastern (Iraqi-born) immigrants as compared to Swedish-born controls. As reported in ***Paper I***, elevated Pro-NT is a predictor of CVD in both genders and T2D in women specifically. The comparatively elevated Pro-NT levels can partly explain the increased T2D risk in Iraqi population, however sex-specific associations of Pro-NT with T2D were not observed in this study as previously observed in MPP.

Wendell *et al.* reported a much higher T2D risk in Middle Eastern immigrants than in native Nordic populations, with the highest risk being observed in women. This indicates that gender differences exist in the T2D risk, as visible in Middle Eastern immigrant women.(71) Furthermore, a modifying effect of Middle Eastern origin on indices of glucose regulations, as measured by associations between higher Pro-NT levels and insulin action, insulin secretion and HbA1c in Iraqi-born population compared to Swedish-born population, were identified. Considering the modest sample size of this observational study, we could not study any gender difference, but the effect of elevated neurotensin in groups of different origin has more significant impact on T2D risk than the differences in neurotensin according to gender.

It was previously reported in the MEDIM study that the Iraqi-born population has comparatively lower insulin sensitivity and higher HbA1c during a non-diabetic stage.(66) Similarly, insulin action is also impaired in the Iraqi-born as compared to Swedish-born controls. As a result, insulin secretion as measured by "deposition index" could not match the degree of insulin resistance resulting in hyperglycemia as shown by higher glycosylated hemoglobin (HbA1c) levels in the Iraqi-born population.(107, 108) Traditional risk factors like age, sex, BMI, and physical activity could not explain the impaired glycemic controls in Iraqi-born population.

As a fat-rich diet increases the secretion of Pro-NT, it is speculated that a high-fat diet explains the elevated Pro-NT levels in the Iraqi-born population. On the other hand, modifying effect of the country of birth on Pro-NT and glucose regulation cannot be fully explained by fat-rich diet alone. It is still not fully clear if there are differences in the country of birth associated modifying effect in the processing of Pro-NT to mature neurotensin hormone that in turn is responsible for the observed correlations. Alternatively, these altered sensitivity effects on the neurotensin receptor level could be due to genetic differences in Iraqi-born immigrants and Sweden born populations.

5.2 Pro-NT, lipid digestion and obesity

Obesity and hyperlipidemia have been shown to be associated with metabolic disorders. Results of ***Paper III*** showed that there is a significant association between the rise of plasma Pro-NT (AUC) and plasma triglycerides (AUC) after an oral lipid load in humans. As previously published animal studies have shown a possible role of NT in intestinal fat absorption, our study contributes to the possible explanation of NT induced fat absorption in humans.

Pro-NT facilitates fat absorption in the small intestines and food rich in fats are potent stimulators of Pro-NT secretion.(20) In the animal studies, NT-deficient mice were protected from obesity, hepatic steatosis, and insulin resistance after high-fat diet, thereby showing the result of significant reduction in Pro-NT induced intestinal fat absorption.(20) In humans, Pro-NT levels were lower in individuals with morbid obesity than in normal-weight individuals.(109) However, elevated Pro-NT were observed in subjects with obesity and/or insulin resistance. Similarly, subjects with NAFLD without morbid obesity had elevated Pro-NT as compared to those without NAFLD and there was a positive correlation between circulating levels of Pro-NT and the severity of NAFLD in obese and non-obese individuals.(19, 110) Additionally, NT and its receptors have recently been expressed in colitis-associated visceral adipose tissue inflammation and macrophage migration involving IL-6 release.(111) Relationship between high Pro-NT levels and visceral adipose tissue dysfunction, increased fatty acid concentration, systemic low-grade inflammation, and aberrant (ectopic) fat deposition in the liver suggests Pro-NT as a possible biomarker of metabolic impairment in obesity.(112, 113)

5.3 Pro-NT and mortality

In ***Paper IV*****,** our main finding was that elevated fasting Pro-NT predicts ACM and deaths due to CVD, GIT diseases, mental and behavioral diseases, and diseases due to unspecific causes.

The follow-up study from the MDC was conducted for a relatively shorter period when sex-specific associations of Pro-NT and CVD mortality were observed, but only in women.(102) To our knowledge, this is the largest epidemiological study to date exploring the relationship between elevated plasma Pro-NT levels and CSM. The results of our study are in line with previous studies regarding the association of Pro-NT and cardiovascular mortality.(102, 114) However, we have seen robust and significant associations in both sexes during a long-term follow-up.

Pro-NT and its receptors have shown effects on growth and proliferation of various cancers in previous studies (115); however, these associations were not observed in the Malmö cohorts. Similarly, no significant associations were observed between Pro-NT and mortality due to respiratory, endocrine, infection, neurological and urogenital diseases in this study.

5.4 Gender differences

In ***Paper I*****,** Pro-NT independently predicts T2D in elderly women whereas associations of Pro-NT with T2D and CVD risk were previously reported in the middle-aged women.(102)

Regarding the association of Pro-NT and cause-specific mortality, previous studies have shown sex-specific associations of Pro-NT with CVD mortality and mortality due to cognitive impairments in the women.(102, 116) These gender-specific associations of Pro-NT in women were, however, not observed in ***Paper IV*****.**

Due to the modest sample size in ***Paper II*** and ***III***, gender differences could not be studied.

5.5 Methodological consideration

5.5.1 Associations and causal inference

In epidemiological study, "association" can be a measure of relative effects (risk, ratio, odds) or absolute effects (risk difference). Some of the explanations for such associations are that:

1. It is a *true* finding.
2. It is a *by chance* finding.
3. It is observed due to exposure and outcome being linked to a third factor (a *confounder*).
4. It is observed due to systematic error in the study i.e., *bias* in the study design or analysis.

An observational study design is more susceptible to methodological problems as compared to an experimental study design because the effects of *exposure* on the study subjects can only be *observed* and cannot be further explored. Thus, causality cannot be proven. It is important to consider these methodological concerns while interpreting the results.(117)

In 1965, Sir Austin Bradford Hill presented nine "fundamental points" for the determination of causal inference in observed epidemiology, referred as the Bradford Hill Criteria. These are strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy. (118)

Randomized control trial (RCT) is still the golden standard to determine if the association is causal or not as observational studies are often prone to confounding and reverse causation, but it is not always possible to conduct RCT due to the extremely high costs of conducting clinical trials and other ethical or technical issues.

Mendelian Randomization (MR) is another alternative approach that uses genetic variants as instrumental variables to identify causal relations between a biomarker and a disease. If a biomarker is causal in a disease development, then the associated genetic factor to that biomarker will also be associated to the disease. Benefit of these studies are that they are not prone to reverse causality (usually disease states cannot change DNA sequence) and confounding (genetic variation is randomly allocated at meiosis). When a biomarker is identified as causal, then modulating pathways to that biomarker needs to be identified for subsequent drug development target.(119)

5.5.2 Study design

As discussed earlier in the Methods section, ***Paper I*** used data from the MPP and ***Paper IV*** from the MDCS-CC, which are *prospective cohort studies*. In these studies, information from the study population is collected at baseline and subjects are followed until the outcome of interest appears, emigration, or until the end of the study period. Various risk factors precede the outcome of interest and longer follow-up periods make it possible to study diseases with a longer latency period such as T2D and CVD. In the *cross-sectional study* (***Paper II***), population data from MEDIM study was used. A major limitation with this study was lack of causal inference, and the possibility of reverse causality. We demonstrated an association between Pro-NT and impaired glucose regulation, as measured by insulin action, insulin secretion and HbA1c in the Iraqi-born population as compared to a Swedish-born population. However, our ability to draw any firm conclusions regarding potential causal relationships for these associations is not possible.

In the *experimental study design* (***Paper III***), the effects (Pro-NT and triglycerides) of an exposure (oral lipid load) on subjects randomized to treatment are explored after exposure.

5.5.3 External validity

External validity is related to the applicability of the study results to other populations. This is also known as generalizability of the study. Most of the participants in the MPP and MDCS-CC studies are white adults of European-ancestry, but in the MEDIM study Iraqi-born immigrants and Swedish-born residents living in Sweden are included, thus assuring the generalizability of our results to these groups.

In cohort study design with long follow-up times, some of the baseline characteristics are likely to change over the follow-up period. In the Swedish population studies assessing the cardiovascular risk factors over time, there was reduction in smoking prevalence from 56.1 % in 1963 to 11.9 % in 2013.(120) Despite the fact that mean BMI and prevalence of T2D had increased over time, it was observed that total cardiovascular risk factor burden has generally decreased in 50-year-old men living in Sweden over the past 50 years from 1963 to 2013.(120) With the decline in the smoking rates in Sweden over time, it is likely that many participants in MPP and MDCS-CC subjects have likely quit smoking and been treated for high blood pressure. Therefore, we would expect such changes to have biased any associations towards null.

5.5.4 Internal validity

In epidemiological studies, the association between the exposure and the outcome needs to be assessed to make valid conclusions of the results. To have internal validity (i.e., how well the study has been conducted), three possible explanations for the observed association need to be ruled out: 1) Bias; 2) Random Error/chance.; and 3) Confounding.

5.5.5 Bias

In epidemiological studies**,** biases are combinations of systemic errors in design, data, analysis, and methods of a study which can give a mistaken estimate of an exposure effect on the risk of disease.(121) It can undermine the internal validity of the research as it deviates the observed associations from the true value. There are three main types of bias: selection bias, information bias and confounding. Bias resulting from conditioning on common effects results in "*selection bias*" and bias resulting from the presence of common causes of exposure and outcome results in "*confounding.*"(122)

Selection bias

For bias to occur, selection must be related to both exposure and outcome. *Prospective cohort study design* usually has minimum selection bias as subjects are

enrolled before they have experienced an outcome of interest. However, over the period, selection bias occurs because of non-response bias and loss-to-follow-up bias. Non-response bias occurs when participants in the study are systematically different from those who refuse to take part in the study or drop out before the completion of the study. It can be minimized by minimizing non-response. The participation rate in MPP was around 72%, which is high.(123) Moreover, the attendance rate for the MDCS-CC was around 76% which was also quite high. In the MEDIM study, the overall response rate in Iraqi and Swedish-born participants were 49% and 32%, respectively, which is relatively low. However, previously reported data have not shown any differences between participants and non-participants for the prevalence of T2D in the MEDIM study sample indicating minimum non-response bias.(124)

In ***Paper I***, the participants in the MPP study were re-examined in 2002-2006 and thus were survivors of the original MPP study (1974-1992). As a result, they were health selected. However, in contrast to a previous study of middle-aged population, the strong independent associations of Pro-NT with CVD in both gender and with T2D in elderly women in ***Paper I*** suggest that health-selected bias is unlikely to have significant effect on the study results. Additionally, the replication cohort of MPP with a longer follow-up time increases the statistical power of the study.

In ***Paper II*****,** participants (Iraqi-born and Swedish-born) were recruited from the same neighbourhood to minimize the potential effect of socioeconomic bias. Additionally, examinations were conducted within a short period of 2 years to reduce assessment bias.

It can be rightfully argued that the aged populations of MPP and MDCS-CC can be influenced by survival bias and that the subjects have used pharmacological treatments over many years. However, the risk of cardiometabolic disease would also be higher in an aged population.

Information bias

Misclassification of exposure and outcome can result in information bias.

At baseline, fasting plasma samples were drawn and immediately stored at – 80 º C for later analyses and fasting plasma concentration of Pro-NT was measured in the previously stored plasma. The long-term stability of proteins in these samples is not known, however, we believe that a minor loss of proteins over time should not affect the positive associations observed from the results of these studies. In that case, there would be underestimation of the risk. Similarly, method used for the evaluation of Pro-NT has been validated in earlier studies.(87, 125)

In the case of outcome misclassification in ***Paper I,*** the main outcome was incident T2D and CVD. We acknowledge that complete information about different subtypes of T2D was not available, but as all the individuals with previous T2D and CVD

diagnosis in the national or local registers were carefully excluded and the study population included older participants without T2D and CVD, it is most likely that the incident cases were T2D and CVD.

5.5.6 Random Error/chance

Random errors are the unpredictable mistakes that can lead to differences between the observed and the true estimates. The effect of random error and the sample variability can be reduced by larger sample size, and it is documented by lower p-value and narrower CIs.

In ***Papers I-IV***, a p-value of <0.05 is considered significant and this indicates strong evidence against the null hypothesis which means that there is less than 5% probability that the null hypothesis is correct.

5.5.7 Confounding

Confounding is very important consideration in observational studies. A factor is said to be a confounder in an association between an exposure and outcome, if it is:

- associated with the outcome.
- independently associated with the exposure.
- not on the casual pathway of association between the exposure and the outcome.

Confounding can be minimized by various strategies at either design phase, analysis phase of the study, or both. In the study design phase, it can be done by randomization, restriction, and matching. In the analysis phase, methods used are stratification, matched analysis, or adjustments for potential confounders.

In all the studies included in this thesis, adjustments for the potential confounders were carried out and have been described in the method section.

5.5.8 Residual Confounding

Residual confounding is the distortion that remains in the association between an exposure and an outcome after controlling for confounding factors in the design and/or analysis of a study. As a result, these confounding factors are not considered and adjusted in the study design. One common cause in observational studies is the lack of data collection for these factors. In ***Paper I***, data about waist measurement was not available in MPP. Data about BMI and waist was not available in ***Paper III***. In ***Paper I*** and ***Paper IV***, information about treatment with statins was not available in the MPP and MDCS-CC.

5.5.9 Effect modification or interaction

Effect modification means that the impact of an exposure on an outcome or an association between a predictor and an outcome is changed, depending on a third variable. This effect can be explored further by stratifying for the interaction variable and then the effect of the risk factor on the outcome at different levels of interaction variable can be observed. Effect modification was tested as follows:

In **Paper I**, significant interactions between Pro-NT and gender on the outcome of incident diabetes were observed, whereas no significant interactions were observed between Pro-NT and gender on the outcome of incident CVD. In **Paper II**, significant interactions were observed between country of birth and Pro-NT in all three models of glucose regulation. Iraqi origin modified the relationship of Pro-NT and indices of glucose regulations. However, no significant interactions were observed between gender and Pro-NT. In **Paper IV**, no significant interaction was observed between Pro-NT and gender on mortality risk.

5.6 Strengths and Limitations

Strength of our studies include longitudinal data with large sample size and availability of replication cohorts in ***study I, II and IV***. Additionally, these studies are warranted to characterize the role of Pro-NT in cardiometabolic diseases in diverse cohorts and across a broader age range. In ***study II,*** participants were recruited from the same neighbourhood to avoid socioeconomic bias. Furthermore, validated and gold standard techniques were used for assessing insulin sensitivity.

However, it is essential to acknowledge several limitations. In all the studies, fasting Pro-NT was used as a surrogate for NT hormone and ELISA (Enzyme-linked Immuno-Sorbent Assay) was not performed to measure NT.

In ***Paper I,*** participants were selected from the MPP-Reexam cohort that were survivors of the original MPP cohort with baseline examinations in 1974-1992. As a result, they were health selected.

In ***Paper II***, participation rate of Iraqi-born was slightly higher than that of Swedish-born individuals (approximately 40% vs 30%). There were no differences observed between participants and non-participants in the prevalence of T2D as compared to earlier studies, thus it indicates a representative study population. Furthermore, as it was a cross-sectional study design this made it difficult to draw any conclusions regarding causality in this study. However, independent associations between Pro-NT and T2D risk in models adjusted for confounding factors have been reported previously in population-based studies.(102)

In ***Paper III***, the findings are based on a limited number of healthy individuals who differ slightly in background factors such as age, sex, and health status. As there was no prior study to use for calculation of an effect estimate, a formal power calculation was not carried out. Similarly, we were able to show the effect of an oral lipid load on Pro-NT levels and triglycerides.

In ***Paper IV***, participants were predominantly Swedish-born, white European nationals and this limits the external validity of the study to other ethnicities. Furthermore, the mortality rate was rather low in this study as the mean age of population sample at baseline was slightly younger than 60 years, giving lesser number of events in some of the major non-CVD and non-neoplasm causes of death during follow-up.

As the diagnostic information about the cause of death is reported by the treating physicians, some caution in the interpretation of results is very important.

6 Conclusion

The work presented in this thesis was aimed to identify the associations between Pro-NT and risk of CVD, T2D and impaired glucose regulation, as well as diet-induced obesity, and to analyse if high levels of circulating Pro-NT was independently associated with mortality in large population-based cohorts. Furthermore, we aimed to understand the immediate effects of dietary fats on circulating Pro-NT levels and triglycerides, and to measure its effect on glucose regulations in a Middle Eastern migrant population with high burden of T2D.

Based on our findings the following important conclusions can be made:

I. Pro-NT independently predicts the development of CVD in an elderly population in both genders. Furthermore, it predicts T2D in elderly women only.

II. Associations were found between elevated Pro-NT, with a modifying effect of ethnic background, on insulin action, insulin secretion and glycaemic control (HbA1c) in an Iraqi immigrant population that differed from a corresponding native Swedish population. We conclude that the stronger associations between Pro-NT and glucose regulation in Iraqi immigrants than native Swedes can explain part of the excess risk of T2D in the Middle Eastern population.

III. Pro-NT increased sharply after an oral fat load of cream as well as olive oil. Furthermore, the degree of the postprandial rise of Pro-NT was significantly related to the postprandial rise of plasma triglycerides. Our data supports the recent results from animal studies suggesting that Pro-NT contributes to the intestinal absorption of lipids into the blood stream.

IV Pro-NT predicts total mortality risk in a population-based study irrespective of gender. The association between Pro-NT and cause-specific mortality risk was mainly driven by CVD mortality, but elevated Pro-NT also predicted death from digestive, mental, and behavioral diseases, as well as deaths attributed to unspecific causes.

7 Future research

This thesis has explored the associations between Pro-NT with obesity, ethnicity, CVD, T2D and cause-specific mortalities in different population cohorts. The results are of interest as Pro-NT is a small peptide with broad effects on metabolic and cardiovascular disorders.

- In ***Paper I*** and ***IV***, the strong associations of elevated Pro-NT with fatal and non-fatal CVD independent of traditional risk factors in both middle-aged and elderly populations, together with its role in lipid uptake from the gut lumen as explored in ***Paper III*** suggest the importance of Pro-NT to be used as a potential biomarker in early risk stratification. This allows us to classify individuals into low-risk or high-risk categories.

- One can also speculate to explore the effect of orlistat treatment, GLP1 analogues or low-fat diet in the long-term setting and thereby study the reduction in cardiometabolic risk profile in subjects with elevated Pro-NT.

- As Pro-NT interacts with the NT- receptors, the possibility to antagonize the NT/NTSR axis can provide further insights into the processes connecting central and peripheral control of appetite and nutrient (fat) absorption.

- The exploration of effects of medications like GLP-1 inhibitors, SGLT2 antagonists and statins on the neurotensin system in long-term studies.

- Similarly, the emerging role of Pro-NT in obesity encourages further studies to analyse long-term consequences of a pharmacological blockade of the neurotensin system as well as rebound effects after the therapy is ended.

- In one of our upcoming studies, we will first isolate the genetic component of variation of Pro-NT by using Genome Wide Association Studies (GWAS) in the MDCS cohort. A score of gene variants associated with high levels of Pro-NT will then be tested for association to obesity, T2D and coronary artery disease. To control the pleiotropic effects and unmeasured confounding, better and reliable genetic instruments and statistical methods will be used.

8 Popular Science Summary

The prevalence of T2D and obesity is rising in the world, and it has reached epidemic proportions in the last decade. Initially known as the problem of high-income countries, it is now recognized as equally affecting middle- and low-income countries. In a recently published systematic review in the trends of T2D, it is shown that increasing prevalence is mainly due to improved medical treatment and decline in mortality whereas the incidence of T2D was rising from 1990 to the mid-2000 and after that it has been reported as stable or falling in over a third of world's population including Sweden. Public health care education and preventive strategies and awareness campaigns could be contributing factors to the declining T2D incidence in adults.(60)

The overall aim of this thesis is to study Pro-NT and its associations with T2D and cardiovascular diseases in different population cohorts. Most of the previous studies on Pro-NT are done in experimental settings and little is known in humans. We planned to study its relationship with T2D and cardiometabolic diseases.

In ***Paper I*****,** we investigated the association of Pro-NT with T2D and cardiovascular disease in elderly population during long-term follow up. This study was a replication study. A previous study was conducted in a middle-aged population and a risk association of Pro-NT with CVD and T2D was significant only in women. It was interesting to replicate these findings and to observe these associations in the elderly population. The results showed that Pro-NT predicts CVD in both elderly men and women whereas it only predicts T2D in elderly women.

In ***Paper II,*** we investigated the association of Pro-NT with T2D in Swedish-born subjects and an immigrant population of Iraqi-born in Malmö (MEDIM study) who were already at high risk of T2D. Elevated levels of Pro-NT were observed both in the Iraqi- vs Swedish-born group and elevated plasma Pro-NT levels were associated with impaired glucose regulations assessed as insulin secretion and action and HbA1c in the Iraqi-born participants only.

In ***Paper III,*** the acute effects of an oral fat load on Pro-NT and plasma triglycerides and their interrelationship in healthy individuals were investigated. Pro-NT increased sharply after an oral fat load of cream as well as olive oil. Furthermore, the degree of the postprandial rise of Pro-NT was significantly related to the postprandial rise of plasma triglycerides. Our human data supported the recent results from animal studies suggesting that Pro-NT contributes to intestinal

absorption of lipids into the blood stream which could explain some of the possible effect of Pro-NT on plasma lipids which is one of the strongest risk factors for CVD and T2D mortality.

In ***Paper IV***, associations between Pro-NT and all-cause mortality and cause-specific mortality in middle-aged population were studied and showed specific and significant associations with cardiovascular diseases, GIT diseases, mental and behavioral disorders, and death due to unspecific causes. This was one of the most important results as the association of Pro-NT and all-cause as well as cause-specific mortality risk was not explored before.

9 Populärvetenskaplig sammanfattning

En oroväckande ökning av fetma och T2D under de två senaste decennierna är globala hälsoproblem som drabbar både rika och fattiga länder. Den individuella risken att utveckla fetma och dess följdsjukdomar beror på både gener och miljön och möjliga interaktioner mellan dessa. Samtidigt finns det behov av rekommendationer och insatser som syftar till att förbygga förekomsten av fetma och T2D i befolkningen.

Pro-NT är ett tarmhormon som frisätts från mag-tarmkanalen efter fettintag. Studier har påvisat samband mellan Pro-NT och olika patologiska tillstånd som fetma, hjärt-kärlsjukdomar, T2D och cancer.

Denna avhandling bygger på data från tre samhällsbaserade kohorter, The Malmö Preventive Project (MPP), The Malmö Diet and Cancer study-cardiovascular cohort (MDC-CC) och The MEDIM study (impact of Migration and Ethnicity on Diabetes in Malmö).

I avhandlingen fokuserade vi på Pro-NT, som är en stabil prekursor för neurotensin hormon, i relation till dietinducerad fetma, hjärtkärlsjukdomar, dyslipidemi, T2D och glukosreglering i populationer med ursprung i Sverige men även Mellanöstern. Sambandet mellan Pro-NT och total samt orsaksspecifik dödlighet studerades också.

I den första studien identifierade vi positiva signifikanta samband mellan Pro-NT och incident kardiovaskulära sjukdom hos äldre individer och samband mellan Pro-NT och incident T2D endast hos kvinnor efter 5,4 års uppföljning. I den andra studien fann vi ett signifikant tvärsnittssamband mellan ökade Pro-NT nivåer, minskad insulinkänslighet samt minskad insulinsekretion hos irakiska individer jämförda med individer födda i Sverige. I tvärsnittsanalys var högre Pro-NT nivåer associerade med ökad risk att utveckla T2D i irakisk-födda population jämfört med individer födda i Sverige. I den tredje studien identifierade vi att en oral fettbelastning given till friska och normalviktiga individer resulterade i en ökning av Pro-NT nivåer tillsammans med en ökning av plasmatriglycerider. Förändringar i cirkulerande nivåer av Pro-NT korrelerade till förändringarna i plasmatriglyceridnivåerna efter fettintag. I den fjärde prospektiva studien

identifierade vi en ökad dödlighetsrisk associerad med förhöjda nivåer av Pro-NT omfattande dödlighet av alla orsaker och orsaksspecifik dödlighet.

Sammanfattningsvis så visar våra resultat att cirkulerande Pro-NT är en biomarkör som förutsäger risken att utveckla hjärtkärlsjukdomar, samt avspeglar insulinresistens, risken att utveckla T2D, dietinducerad fetma och dödlighetsrisk. Detta kan i sin tur förbättra identifiering av individer med förhöjd risk i ett tidigt skede och därmed förändra sjukdomsförloppet samt tidiga insatser för att minimera risken för kardiometabola sjukdomar.

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Role of Proneurotensin in Cardiometabolic Diseases

Ayesha Fawad is a medical graduate from Pakistan. She completed her medical degree at Fatima Jinnah Medical University, Lahore in 2003 and 3 years of specialist training (ST) in Internal Medicine and Cardiology in Pakistan. After completion of her specialist training (ST) in General Medicine in Sweden, Ayesha is now working as a Specialist in General Medicine in Malmö since 2019.

The focus of this doctoral thesis was to investigate the role of Proneurotensin in the prediction of different health outcomes in the population of Malmö. This thesis shows that elevated levels of Proneurotensin are associated with increased risk of adverse health outcomes and identification of such high-risk individuals earlier on in life can be useful to potentially alter the disease course and prognosis.

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