Prospective Studies of Risk Factors Associated with Type 2 Diabetes, Cardiovascular Disease, and Mortality in Elderly Women

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NORDIC BIOSCIENCE

Ph.D. Thesis

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To the 806 remarkable women

whom I personally met while collecting data for PERF II.

You all had a dream of helping future generations to age well.

Preface

The present thesis is the result of a PhD project carried out from June 2013 to October 2016 under the supervision of Associate professor Susanne Brix Pedersen from the Technical University of Denmark, Kim Henriksen and Morten Asser Karsdal from Nordic Bioscience A/S and Professor Henning Beck-Nielsen from Odense University Hospital.

The work leading up to this PhD thesis can be divided into three parts; clinical study-set up, data collection, and data analysis. I was responsible for the study set-up, protocol approval, along with hiring and training of all staff involved in the study. This initial step was followed by enrolment of 2,103 elderly Danish women within 16 months, meaning 4-6 daily visits in their own home. Alongside data collection the application for register-linkage was approved, and after data cleaning the longitudinal data analysis could be initiated.

The Technical University of Denmark (DTU) has funded the PhD in collaboration with the Danish Research Foundation (Den Danske Forskningsfond).

The thesis is based on four studies represented by either submitted or published manuscripts:

I. Cohort Profile: The Prospective Epidemiological Risk Factor (PERF) Study. JS Neergaard*, **K Dragsbæk***, SN Kehlet, HB Hansen, G Hansen, I Byrjalsen, P. Alexandersen, LM Lindgren, A Bihlet, BJ Riis, JR Andersen, P Qvist, MA Karsdal, and C Christiansen. Published in International Journal of Epidemiology, 2016 Oct, 1–9, Epub ahead of print: doi: 10.1093/ije/dyw251

II. Metabolic Syndrome and Subsequent Risk of Type 2 Diabetes and Cardiovascular Disease in Elderly Women: Challenging the Current Definition. **K Dragsbæk**, JS Neergaard, JM Laursen, HB Hansen, C Christiansen, H Beck-Nielsen, MA Karsdal, S Brix, and K Henriksen. Published in Medicine (Baltimore) 2016 Sep;95(36):e4806.

III. Weight Change and Risk of Hyperglycaemia in Elderly Women. **K Dragsbæk**, JS Neergaard, C Christiansen, MA Karsdal, H Beck-Nielsen, S Brix, and K Henriksen. In review, prepared for re-submission to Aging Clinical and Experimental Research.

IV. Matrix Metalloproteinase Mediated Type I Collagen Degradation – An Independent Risk Factor for Mortality in Women. **K Dragsbæk***, JS Neergaard*, HB Hansen, I Byrjalsen, P Alexandersen, SN Kehlet, AC Bay-Jensen, C Christiansen, and MA Karsdal. Published in EBioMedicine 2015 Apr 30;2(7):723-9.

*joined first-authors.

Moreover, I have contributed to five manuscripts during the PhD, which are not integrated in the thesis:

1. Late-Life Risk Factors for All-Cause Dementia and Differential Dementia Diagnoses in Women: A Prospective Cohort Study. JS Neergaard, **K Dragsbæk**, HB Hansen, K Henriksen, C Christiansen, and MA Karsdal. Published in Medicine (Baltimore). 2016 Mar;95(11):e3112.

2. Subtypes of Mild Cognitive Impairment and Progression to Dementia: The Prospective Epidemiological Risk Factor Study. JS Neergaard, **K Dragsbæk**, K Henriksen, C Christiansen, and MA Karsdal. Journal of Alzheimer's Disease, submitted October 2016.

3. Two Novel Serum Biomarkers Measuring Degradation of Tau with Prognostic Utility for Preclinical Dementia. JS Neergaard, **K Dragsbæk**, C Christiansen, MA Karsdal, S Brix, and K Henriksen. In review, PloS Medicine, submitted September 2016.

4. Remodeling of the tumor microenvironment predicts increased risk of cancer in postmenopausal women - The Prospective Epidemiologic Risk Factor (PERF I) Study. CL Bager, N Willumsen, SN Kehlet, HB Hansen, AC Bay-Jensen, DJ Oersnes-Leeming, **K Dragsbaek**, JS Neergaard, C Christiansen, E Høgdall, and MA Karsdal. Published in Cancer Epidemiol Biomarkers Prev; 25(9); 1348–55.

5. Excessive matrix metalloprotease-mediated degradation of interstitial tissue (type I collagen) independently predicts short-term survival in an observational study of postmenopausal women diagnosed with cancer. N Willumsen, CL Bager, SN Kehlet, **K Dragsbæk**, JS Neergaard, HB Hansen, AC Bay-Jensen, DJ Leeming, A Lipton, C Christiansen, M Karsdal. In review, re-submitted to Oncotarget, September 2016.

Abstract

The world's population is ageing. With an increased life expectancy across the globe, more people will live into old age. Women outlive men averagely by four years, warranting an increased focus on healthy ageing in women. The demographic shift resulting in an increased fraction of elder individuals has given rise to concerns about whether the extra life years added are spent in good health or with disease conditions resulting in high impacts on health care systems, socioeconomic relations and on the individual level. The World Health Organization predicts the burden of non-communicable diseases such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) to account for more than three-fourths of the total disease burden in middle and high-income countries before year 2030. Despite the identification of many risk factors for non-communicable diseases within the last decades, these risk factors remain the leading contributors to decreased healthy life expectancy in late-life, necessitating an increased focus on risk factors and non-communicable diseases specifically in elderly.

The studies constituting the foundation of this thesis aim to explore hypotheses focusing on known and novel risk factors and their relation to ageing, disease, and mortality in elderly Danish women. The studies are epidemiological in their character and based on data from the Prospective Epidemiological Risk Factor (PERF) study, a community-based cohort study on 5,855 elderly Danish women enrolled in year 2000 with a follow-up examination of 2,103 of the women in year 2013 (study I).

Data from the PERF cohort was used to evaluate whether the metabolic syndrome (MetS), a cluster definition of cardio-metabolic risk factors, is a valid and useful tool for prediction of future T2DM and CVD specifically in elderly women (study II). The study described how women fulfilling the current MetS criteria set by the International Diabetes Federation revealed an increased risk of future T2DM or CVD diagnosis. However, subjects who did not fulfil the definition criteria for MetS, but presented one or more of the MetS risk factors were likewise at increased risk. A further subdivision of the control group showed to increase the risk of T2DM to 6.3-fold (from 3.6-fold) and 1.7-fold for CVD (from 1.3-fold) for MetS-defined women when compared specifically to a control group solely including women with no MetS risk factors. Based on these risk estimates, it was concluded that employment of the MetS in elderly women should be focused only as a tool for identifying subjects with metabolic high-risk profiles. Further, the sum of risk factors was proposed to be equally considered, as elderly women holding only a few MetS risk factors, were also at increased risk of T2DM and CVD.

The cohort was further used to explore how weight and weight change in late-life affected the risk of hyperglycaemia in elderly women (study III). The study presented a 2-fold increased risk of hyperglycaemia in overweight and obese elderly women compared to normalweight women after 13 years. In women who gained weight, the risk of hyperglycaemia in late-life was most profound for overweight and obese women resulting in a 2.7-fold increased risk of hyperglycaemia in overweight weight gainers and a 3.2-fold increased risk in obese weight gainers compared to normalweight weight-stable women. Contrarily, overweight and obese women who lost weight during the follow-up period decreased their risk of hyperglycaemia to a level comparable to women who stayed normalweight during the follow-up period.

The thesis rounds off by introducing a novel risk factor; matrix metalloproteinase (MMP)-mediated degradation of collagen type I (C1M) that was used in the description of mortality in elderly women (study IV). The study showed how increased MMP-mediated tissue degradation, as an independent risk factor, was associated with a 2-fold increase in all-cause mortality within three years of follow-up and a 1.5-fold increase in all-cause mortality up to nine years prior to death.

Overall, these studies contribute to the knowledge specifically demanded on women's health in late-life by describing associations between known risk factors of the MetS and subsequent risk of T2DM and CVD. Further, by highlighting associations between hyperglycaemia, weight and weight change in late-life, and lastly by the evaluation of collagen type I degradation possibly being an important predisposition for increased mortality in elderly women.

Dansk Resumé

Verdens befolkning bliver ældre. Den globale forøgelse i middellevetid gør, at flere mennesker vil leve til de bliver gamle. Kvinder lever i gennemsnit fire år længere end mænd, hvorfor et øget fokus på netop kvinders aldring er specielt berettiget. Ændringen i den demografiske fordeling, med en større andel af ældre, har ledt til bekymring om, hvorvidt de ekstra leveår vil være sygdomsfrie eller om aldring vil være ledsaget af sygdom. Hvis de ekstra leveår resulterer i en øget sygdomsbyrde, vil det have store konsekvenser for sundhedssystemer og socioøkonomiske forhold, men også specifikt for den ældre borger. Verdenssundhedsorganisationen, WHO, estimerer, at andelen af ikke-smitsomme livsstilssygdomme såsom type 2 diabetes mellitus (T2DM) og hjertekarsygdomme (CVD) vil udgøre tre fjerdedele af den totale sygdomsbyrde i middel- og højindkomstlande inden år 2030. På trods af omfattende kortlægning af mange af de risikofaktorer, som har vist sig at lede til livsstilssygdomme, er disse fortsat medvirkende til at aldring ikke er en sygdomsfri proces. Dette nødvendiggør et øget fokus på risikofaktorer og livsstilssygdomme specielt hos ældre mennesker.

Studierne, der udgør grundlaget for denne afhandling, har til formål at undersøge risikofaktorer og deres relation til aldring, sygdom og dødelighed i ældre danske kvinder. Arbejdet udgøres af epidemiologiske studier og er baseret på data fra det Prospektive Epidemiologiske Risikofaktor (PERF) studie, et kohortestudie af 5855 ældre danske kvinder, inkluderet i år 2000, med et opfølgende besøg for 2103 af kvinderne i år 2014 (studie I).

Data fra PERF kohorten er i denne afhandling blevet brugt til at undersøge, om det metaboliske syndrom (MetS), en definition, der bruges til at beskrive de største kardio-metaboliske risikofaktorer, er et brugbart værktøj til at forudsige risikoen for T2DM og CVD i ældre kvinder (studie II). Studiet beskriver, hvordan de kvinder, der opfylder det nuværende kriterie for MetS, som er fastsat af den Internationale Diabetes Føderation, viste sig at have en øget risiko for fremtidig T2DM eller CVD. Det viste sig dog også, at kvinder, der ikke opfyldte kriterierne for MetS, men som stadig havde en eller flere af de metaboliske risikofaktorer, ligeledes viste sig at have en øget risiko for T2DM og CVD. En yderligere opdeling af kontrolgruppen viste, at risikoen for T2DM hos kvinder med MetS blev øget til 6,3 fold (fra 3,6 fold) og 1,7 fold for CVD (fra 1,3 fold) sammenlignet med kontrolgruppen, hvis denne gruppe udelukkende bestod af kvinder uden nogle MetS risikofaktorer. Baseret på disse fund, blev det konkluderet, at brugen af MetS udelukkende bør bruges som et redskab til at identificere kvinder med metaboliske højrisikoprofiler. Ydermere blev det konkluderet, at summen af risikofaktorer

ligeledes skulle vurderes hos kvinder, der ikke falder ind under det nuværende MetS-kriterie, men som stadig har én eller flere risikofaktorer for MetS, da disse kvinder ligeledes blev identificeret til at være i øget risiko for T2DM og CVD.

Kohorten blev yderligere anvendt til at undersøge, hvorledes vægt og vægtændring sent i livet påvirkede risikoen for hyperglykæmi hos ældre kvinder (studie III). Studiet viste en 2-fold forøget risiko for hyperglykæmi hos overvægtige og svært overvægtige ældre kvinder sammenlignet med normalvægtige kvinder ved opfølgning efter 13 år. Risikoen for hyperglykæmi sent i livet var størst for overvægtige og svært overvægtige ældre kvinder, som havde taget på i opfølgningsperioden. Vægtøgning resulterede i en 2.7-fold øget risiko for hyperglykæmi hos overvægtige kvinder og en 3,2-fold øget risiko hos svært overvægtige kvinder sammenlignet med normalvægtige kvinder med stabil vægt. Modsat fandt vi, at overvægtige og svært overvægtige kvinder, der tabte sig i perioden mellem første og andet besøg, havde en nedsat risiko for hyperglykæmi, svarende til niveauet set i kvinder, der forblev normalvægtige i opfølgningsperioden.

Afhandlingen afrundes med introduktionen af en hidtil ukendt risikofaktor, matrix-metalloproteinase (MMP)-medieret nedbrydning af kollagen type I (C1M), i beskrivelsen af dødelighed hos ældre kvinder (studie IV). Studiet viste, hvordan øget MMP-medieret vævsnedbrydning, som en uafhængig risikofaktor, var forbundet med en fordobling i risiko for død inden for tre års opfølgning og en 1,5 fold stigning i risiko for død op til ni år før døden indtraf.

Samlet set bidrager studierne i denne afhandling til at afdække kravet om specifik viden inden for ældre kvinders sundhed ved at beskrive associationer mellem kendte risikofaktorer for MetS og deraf øget risiko for T2DM og CVD. Endvidere fremhæves risikoen for hyperglykæmi sent i livet som et resultat af vægt og vægtændring, og endelig ved evalueringen af MMP-medieret nedbrydning af kollagen type I som en mulig risikofaktor for øget dødelighed hos ældre kvinder.

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Finally, the biggest thanks of all is to you, **Mads.** You showed me the true essence of what makes life balanced. For that, I am forever grateful. Thank you!

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List of Abbreviations

ADA	American Diabetes Association
BMI	Body Mass Index
C1M	Collagen type 1 degradation
CVD	Cardiovascular Diseases
FFA	Free Fatty Acids
FPG	Fasting Plasma Glucose
HDL	High-density lipoproteins
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IL-6	Interleukin-6
MetS	Metabolic Syndrome
OGTT	Oral Glucose Tolerance Test
PCA	Principal Component Analysis
PERF	The Prospective Epidemiological Risk Factor Study
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumour Necrosis Factor Alfa
VLDL	Very Low Density Lipoproteins
WHO	World Health Organization

1 PROLOGUE

Every generation grows older than the previous, and this increased probability of survival into old age is one of humanity's major achievements in global health (1). It is anticipated, that the worldwide population will encompass more old people than children before the year 2030 and this demographic shift is majorly a result of decreasing fertility, advances in medicine, and socioeconomic development (2). As both the length of life but also the proportion of elderly people will increase, key concerns arise. Will the increased life span lead to increased longevity and a longer life in good health, or be accompanied by illness, frailty, and increased dependency? The health of the elderly population and the term 'healthy ageing', is consequently of greater interest than ever.

Knowledge of disease and health is largely built upon population-based studies of the distribution and determinants of disease outcomes (3). The scientific discipline of epidemiology is used to assess if obtained data is consistent with current scientific knowledge and hypotheses, and to describe the natural history of diseases (3,4). The studies in this thesis are based on observational non-experimental epidemiology assessing risk factors associated with distortion of healthy ageing in elderly women. More specifically, the focus of this thesis is both on well-known and novel risk factors associated with increased risk of type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD) and mortality analysed in a cohort of elderly Danish women. The women were enrolled in the Prospective Epidemiological Risk Factor (PERF) study in year 2000 with a follow-up visit in year 2013.

2 INTRODUCTION

The world's population aged ≥65 years is estimated to increase from currently 524 million to 1.5 billion by year 2050 (5,6). The rising life expectancy within the older population is increasing the number and proportion of people at old ages, which has resulted in a global population where 12% are ≥65 years (7). If focusing specifically on the industrialized parts of the world, Europe has the largest percentage of elderly people, with 24% of the total population being ≥65 years (8). Further, within the industrialized countries, the oldest old, which account people aged ≥85 years, constitute 12% of the elderly population and they are now the fastest growing segment of the population. On a global scale, the oldest old are projected to increase by 351% before year 2050, followed by the ≥65 segment increasing 188% between year 2010 and 2050. In comparison, the population aged <65 will merely increase with 22% (5,9). This demographic shift has given rise to concerns regarding the decrease in eligible workforces, demands for later retirement age, and costs of poor health of the elderly population (7).

Traditionally, a decline in mortality reflected an equal decline in morbidity. However, in the industrialized parts of the world, where mortality rates have been continuously decreasing throughout the decades, the advances in life expectancy are largely caused by mortality reductions from chronic diseases at older age. This has raised doubts on whether a longer life also means better health for the surviving elderly population (10). The study of ageing as a discipline thus largely focuses on chronic diseases in late-life, and how the population of study is affected as a result of modifiable risk factors, e.g. obesity, physical inactivity, diet, smoking etc. together with non-modifiable risk factors, e.g. gender, ethnicity, and family predisposition (11). Strategies applied to prevent distortion of a healthy ageing process largely focuses on modifiable risk factors, which will also be the focus of the following parts of this introduction.

2.1 Ageing and Disease

The shift in ageing patterns has resulted in a change in the leading causes of disease and death. Non-communicable diseases, such as CVD, cancer, Alzheimer's disease and T2DM, have largely overtaken infectious and parasitic diseases as the leading health threats. The Global Burden of Disease project estimates that among the elderly population, non-

communicable diseases already account for more than 87% of the disease burden worldwide, with CVD resulting in 46% of all deaths globally in elder women (11,12). Women specifically, as they live longer than men, represent a growing proportion of all older people. Global estimates from the World Health Organization (WHO) report 55% of all adults aged 60 years or older to be women, a proportion that rises to 58% at the age of 70 and above (13). Much of the burden of disease that women face could be prevented by addressing the six most critical risk factors for chronic disease: Hypertension, hyperglycaemia, dyslipidaemia, overweight/obesity, physical inactivity, and tobacco use. These modifiable risk factors account for 63% of all deaths from CVD and T2DM and over 75% of all deaths from ischaemic heart disease in women (14,15). WHO has on this note demanded longitudinal studies, which incorporate measures specifically focusing on the health of the elderly population in order to understand and prevent non-communicable age-related diseases, and to focus existing knowledge about prevention and treatment specifically in this fast growing segment of the population (5).

2.2 Obesity

The increase in life expectancy together with the increased prevalence and severity of obesity signifies a double disease burden for the future (16). Obesity, in particular abdominal obesity, is a strong risk factor for the development of cardiovascular outcomes such as elevated blood pressure, low levels of high-density lipoprotein (HDL)-cholesterol, elevated triglycerides and disturbed glucose metabolism (17). This makes abdominal obesity a well-known and independent risk factor for both T2DM (18) and CVD (19,20). Obesity is therefore often referred to as the predecessor of other risk factors known to result in non-communicable diseases (21).

2.2.1 Obesity prevalence

Overweight, defined as a body mass index (BMI) of 25-29.9kg/m^2, is by the WHO estimated to affect 1.3 billion people and a further 600 million people are classified as obese with a BMI ≥30kg/m^2 (21). The prevalence of overweight and obesity in the adult population is predicted to rise from the 33% reported in 2005 to 57.8% in 2030 if the current trends in obesity development continue (22).

Focusing specifically on obesity prevalence in the elderly age group, the reported prevalence vary. Data from the Behavioural Risk Factor Surveillance System in the US

provided BMI data in 2003 on 52,921 elderly individuals aged 65 years and older. They identified 20.3% elderly classified as obese. In the 65-74-year age group, 25% of the elderly had a BMI of ≥30 kg/m², which was significantly higher than the 16.6% in the 75-84-year age group. The prevalence of obesity in the ≥85-year age group was 9.9% (23). Another US study, the follow-up of the National Health and Nutrition Examination Survey (NHANES II), conducted in 2007–2010, indicated that more than one-third of older adults, aged ≥65 years, were obese (24,25). The prevalence of obesity was greater in subjects aged 65–74 (40.8 %), compared to those aged ≥75 years (27.8 %) in both men and women (24,25). European data likewise indicate that the prevalence of obesity in the elderly age group will continue to increase, however, varying magnitudes are reported in different European studies. The Scottish Health Survey showed that between 1998 and 2008, the overall prevalence of obesity showed little increase overall, however, the BMI continued to rise between age 60 and 70, especially in women (26). In the French Obésité Epidémiologie survey with data from 1997 to 2006 an obesity prevalence of 17.9% was reported for the elderly aged ≥65 years. In even older ages, the prevalence decreased from 19.5% in the 65-69-year-olds to 13.2% in the oldest old aged ≥80 years (27). Further, the European Prospective Investigation on Cancer and Nutrition with participants aged 40-65 years in 1996 predicted a prevalence of obesity of 30% in 2015 in a linear prediction model and 20% in a levelling off model (28). Other longitudinal cohort studies equally demonstrated body weight and BMI values decreasing slightly in older adults (29–31). The proposed levelling prediction model has recently been confirmed in the pan-European survey of obesity conducted by Gallus et al. reporting a European prevalence of 18% obese elderly aged ≥65 years (32).

In Denmark, 40% of all women are overweight or obese according to the Danish National Health profile from 2013. In women aged ≥65 years 49.8% are overweight or obese (33).

2.2.2 Obesity and ageing

Ageing is associated with central changes in metabolism and body composition resulting in a decrease in fat-free mass of up to 40%, in return for an increasing fat mass (34–36). The body fat distribution also changes with age, with visceral abdominal fat increase and subcutaneous abdominal fat decrease (37–39). After the age of 70, both fat-free mass and fat mass decrease in parallel with fat increasingly being deposited in skeletal muscle and in the liver resulting in an increased insulin resistance. This is more evident in women than in men (36).

The change in fat distribution resulting in central and visceral obesity have been shown to be more pro-inflammatory than global obesity and the inflammatory burden is therefore larger in obese elderly compared to normalweight elderly (40). The presence of inflammatory markers such as tumour necrosis factor alfa (TNF-α) and interleukin-6 (IL-6) are known to have catabolic effects on muscle mass, which are involved in the development of sarcopenia and a decrease in fat free mass (34,35,41,42). This has given rise to various studies within sarcopenic obesity studying the loss of muscle mass in return for increased body fat (42–44). The low-grade inflammatory state described in ageing is, besides the association with decreased lean body mass, also reflected in reduced immune function, cognitive decline, and insulin resistance (45).

Obesity and ageing are, besides changes in body composition, also characterized by endocrine changes (41). These changes include alterations in gonadal steroids and thyroid hormones comprising a decrease in growth hormone and testosterone, following an impaired sensitivity to thyroid hormone and leptin, altering satiety (35,36). Further, the changes in hormones that occur with normal ageing are amplified in the presence of abdominal obesity and insulin resistance (34).

2.2.3 Obesity pathophysiology

Obesity pathophysiology and the implications on healthy ageing starts with an understanding of adipose tissue biology (Figure 1).

Adipose tissue modulates the metabolism by releasing free fatty acids (FFA), hormones and pro-inflammatory cytokines (46–48) and in obese subjects, the production of many of these mediators are increased. The abdominal adipose tissue produces numerous inflammatory cytokines such as IL-6 and TNF-α, while the production of adiponectin, the anti-inflammatory adipokine, is diminished (49,50). Adiponectin has been found to protect from insulin resistance and CVD (51), whereas FFA and pro-inflammatory mediators promote the development of insulin resistance (46,52,53).

2.2.4 Insulin resistance and obesity

The ability of insulin to regulate circulating FFA and glucose uptake, by mediating disposal into skeletal muscle, inhibiting gluconeogenesis in the liver (54), and its ability to suppress lipolysis in the adipose tissue, is generally referred to as insulin sensitivity (55). In healthy subjects, there is a feedback loop between the insulin sensitive tissues and the insulin

producing beta-cells in the pancreas, as they increase insulin supply in response to demand by the muscles, liver, and adipose tissue (56). A failure of this feedback loop underlies the development of diabetes as it will result in a deviating glucose tolerance (57). The result of this beta-cell dysfunction and inadequate insulin secretion is an increase in fasting glucose levels owing to incomplete suppression of hepatic glucose production and decreased efficacy of liver and muscle glucose uptake. The elevated FFA levels is a second metabolic component contributing to the gradual loss of beta-cell function (57). This dual effect is referred to a glucolipotoxicity and links obesity with insulin resistance and T2DM. This leads to a lower glucose uptake in skeletal muscle, increased levels of FFA, less inhibition of hepatic glucose production, and subsequent hyperglycaemia (55).

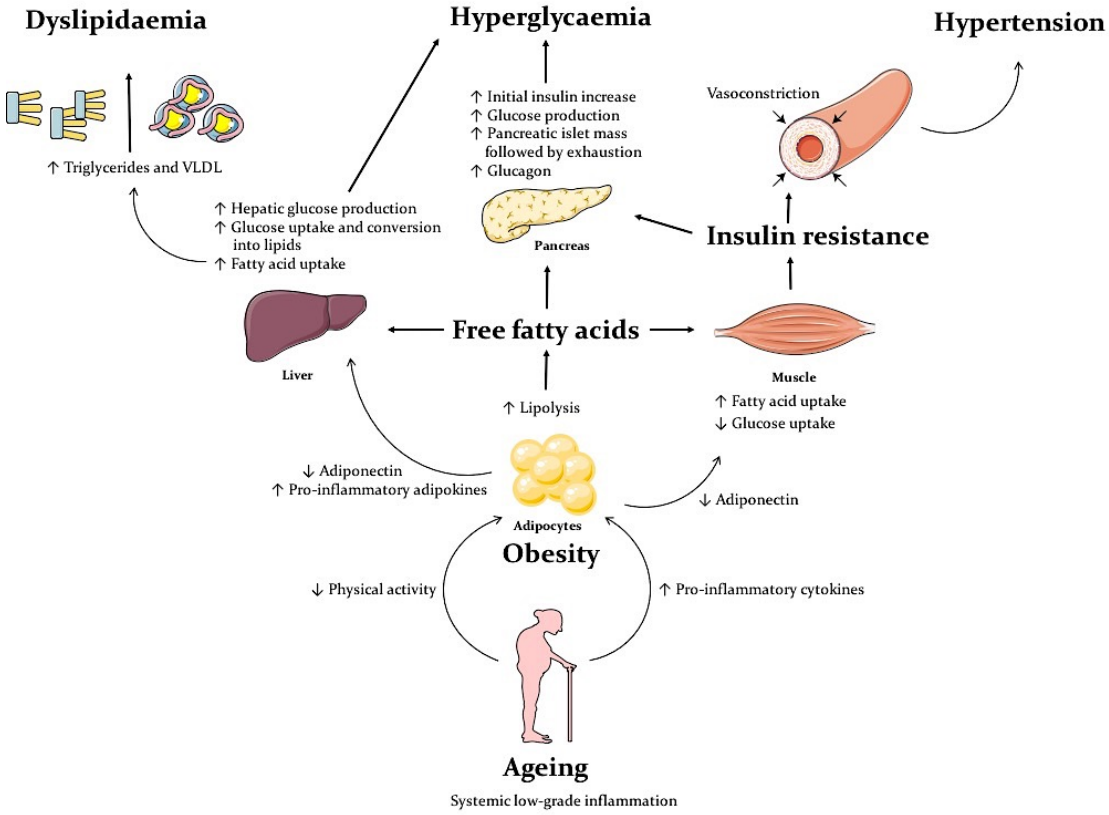

Figure 1 Ageing, obesity and their associations to insulin resistance and metabolic outcomes.

The release of FFA is a critical factor in modulating insulin sensitivity and increased FFA levels are observed in both obese and T2DM subjects and are associated with the insulin resistance observed in both conditions (58,59). Because insulin regulates both the FFA released from adipose tissue triglycerides and the FFA released as a result of lipoproteins undergoing lipolysis, the development of insulin resistance leads to an enhancement of both

FFA release and impaired lipoprotein clearance (60). This consequently leads to lipid deposition and lipotoxicity in ectopic sites, such as liver, skeletal muscle and pancreatic islets (61). Alongside this knowledge of the negative effects of excess lipids, the overflow hypothesis has been proposed by R.H. Unger in 2003 (62) describing how an excess energy intake causes overloading of adipose tissue. The capacity to store FFA in adipocytes is exceeded, and lipids accumulate in ectopic sites, which thereby causes insulin resistance.

Insulin sensitivity fluctuates during the life cycle with an increase in insulin resistance observed with ageing (63). An explanation for this increased resistance in ageing is the impaired mitochondrial function and reduced cellular energy supply believed to result from defects in mitochondrial oxidative phosphorylation, as this was found to be related to lipid accumulation in the muscle of elderly patients (64,65).

2.3 Metabolic Consequences of Insulin Resistance

It is now generally accepted, that insulin resistance plays an important role in the clustering of risk factors associated with CVD and diabetic outcomes (66). The ageing-associated change in body composition further promotes this insulin resistance and in the following sections, some of the most common metabolic risk factors related to an increase in fat mass are elucidated.

2.3.1 Hyperglycaemia

Most obese, insulin resistant subjects will never develop hyperglycaemia as the beta-cells increase the release of insulin sufficiently to overcome the decreased efficiency of insulin action, which will thereby maintain normal glucose levels (56,67). In order for obesity and insulin resistance to be linked with hyperglycaemia and T2DM, the insulin-producing beta-cells must be unable to compensate for the decreased insulin sensitivity (68). Most often the ability of the beta-cells to produce enough insulin subsequently declines over time in insulin resistant subjects (69). When beta-cell dysfunction is a reality, impaired glucose tolerance (IGT), impaired fasting glucose (IFG), and finally T2DM result (57). The transition from the two asymptomatic pre-diabetic stages, IGT and IFG, to full T2DM development may take many years (70).

The 2-hour plasma glucose concentration after an oral glucose tolerance test (OGTT) was recommended by WHO in 1980 (71) to determine IGT. The American Diabetes Association (ADA) introduced IFG in 1997 and WHO defined the term in 1999 with the

purpose of classifying subjects who had fasting glucose levels between normal and diabetic levels (72,73). The lower limit for this classification was decreased from 6.1mmol/L to 5.6mmol/L by ADA in 2004 (74). The two types of hyperglycaemia are proposed to reflect different types of insulin resistance (75–78).

The prevalence of hyperglycaemia is increasing and projected to affect 470 million people by 2030 (79). Estimates indicate that up to 70% with pre-diabetic characteristics eventually will develop diabetes (80,81). The meta-analysis conducted by Levitan et al. in 2004 (82) assessed the risk associated with blood glucose levels in the non- and pre-diabetic range on CVD outcome. They found an increased risk associated with blood glucose levels in the pre-diabetic range, however, they do highlight how the different methods of glucose assessment may have contributed to the heterogeneous results in the published studies. This uncertainty is equally highlighted by Nathan et al. (70) questioning whether the pre-diabetic states convey the risk or if it can be attributed to the development of diabetes during follow-up.

It is not known whether elevated blood glucose levels in the elderly are associated with the same risk as in middle-aged subjects and whether the different types of hyperglycaemia (IGT or IFG) are equally harmful at all ages (83).

2.3.2 Hypertension

Hypertension is very common in the older population, affecting up to 50% of all persons aged ≥65 years (36). The hemodynamic effects of insulin serve under normal conditions as a powerful vasodilator (84). When insulin resistance is present, the vasodilatory effect of insulin is lost, which will lead to endothelial dysfunction following an increased risk of hypertension (85–87). However, the association has been found to be less strong after adjustment for body mass index (88,89) suggesting other factors directly related to adipose tissue plays an additional role (90). It is well known that the incidence of CVD is increased in hypertensive patients, even when the hypertensive state is treated (91,92).

2.3.3 Dyslipidaemia

The increased flux of FFA to the liver in obese individuals increases hepatic production of triglyceride-rich very low density lipoprotein (VLDL)-particles (60). Furthermore, decreased levels of phospholipids, which are necessary for HDL-particles to form, result in reduced breakdown of the VLDL-particles. An elevated level of VLDL-particles in the plasma thereby

result in an increased concentration of triglycerides in the HDL-particle and a decreased cholesterol concentration (93). The triglyceride-rich HDL-particles are small and dense and therefore quickly cleared by the liver, which is manifested in lowered HDL-cholesterol levels (94). Hypertriglyceridemia and HDL-cholesterol levels are the main lipid disturbances describing the metabolic syndrome (MetS).

A substantial proportion of older adults are dyslipidaemic, including the oldest old, aged 80 years and older (95). In women, the increase in LDL-cholesterol and decrease in HDL-cholesterol levels seen during menopause, has been speculated to be the main reason for the increase in CVD incidence reported in women after menopause (96). It has been established that in older adults, dyslipidemia often coexists with obesity, T2DM, and hypertension (95).

2.4 Metabolic Syndrome and Metabolic Diseases

In 1988 G.M. Reaven suggested the existence of a syndrome, *syndrome X*, describing the co-occurrence of a number of metabolic disorders such as hypertension, hypertriglyceridemia, low HDL-cholesterol, and hyperglycaemia (97). Insulin resistance was suggested as the link connecting the occurrence of these abnormalities, thus giving rise to the name, *the insulin resistance syndrome*. Further knowledge on the disproportionate flux of FFA from excess adipose tissue, and how this was believed to be a central component in the development of the syndrome, has later led to the term, *the metabolic syndrome*. The overall goal of applying a definition was to identify individuals at greater risk of developing T2DM and CVD.

2.4.1 Definitions of the metabolic syndrome

Several definitions and explanations of the syndrome have emerged based on the outcome of interest (T2DM, CVD or both). The MetS definition proposed by the WHO in 1998 was developed as a tool to be applied to both diabetic and non-diabetic subjects as it formed part of a consultation report on the definition, diagnosis and classification of diabetes mellitus and its complications (98). The WHO definition included a pre-requisite for either glucose intolerance (defined as either IFG, IGT or diabetes) or insulin resistance (measured using the hyperinsulinemic-euglycaemic clamp technique). The WHO definition was the first guideline which enabled comparability between studies. The WHO definition technically required subjects to undergo an OGTT, clamp studies and measurement of microalbuminuria in supplement to at least two of the metabolic risk factors; obesity,

hypertension, and dyslipidaemia. In an epidemiological context usually including large scale data, the OGTT and insulin resistance measurement was impossible, and not commonly performed. Most studies employed the surrogate calculation of insulin sensitivity using the homeostatic model assessment (HOMA-IR) instead to quantify assessment of the contributions of insulin resistance to the fasting hyperglycaemia (99).

In 1999 the European Group of the Study of Insulin Resistance (EGIR) followed WHO with a definition only to be applied to non-diabetics. The EGIR definition thereby acknowledged the lack of rationale associated with identifying those with the MetS who already had T2DM, in relation to risk prediction as the main goal (100). In addition, as a result of the difficulties in comparing studies using the clamp technique to measure insulin resistance, the EGIR version recommended that insulin resistance was defined as the top quartile of fasting insulin values in the non-diabetic population of study. A standardized cut-off point for insulin measurement was not believed to be possible, due to the different standards for assaying insulin. Obesity was defined by waist circumference, new cut-off values were proposed for hypertension and dyslipidaemia, and microalbuminuria was disregarded (100).

A more recent definition of the syndrome was presented in the Summary of the Third Report of the U.S. National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (101). The NCEP-ATP III definition of the MetS was designed to be applied in clinical practice. A simplified structure of the definition included diagnosis with any three of the five metabolic risk factors, and no requirement for an OGTT or insulin resistance measurement was included, reflecting the more clinically focused proposal.

Finally, the latest MetS definition was proposed by the International Diabetes Federation (IDF) in April 2005 focusing on large waist circumference as the entrance criteria for a further definition of the syndrome (102). The IDF proposed a worldwide definition, with ethnicity-specific cut-points for overweight. The components were identical to those used by the NCEP-ATPIII, but with large waist circumference as a prerequisite component. The WHO, EGIR, NCEP-ATPIII and IDF definitions are summarized in Table 1.

Table 1 Four definitions of the metabolic syndrome in Caucasian women (98,100–102).

WHO criteria World Health Organization **1998**	**EGIR criteria** European Group of the Study of Insulin Resistance 1999
Impaired regulation of glucose identified by either: - T2DM - IFG (fasting glucose ≥6.1mmol/L) - IGT (OGTT) - Lowest quartile of glucose uptake using the hyperinsulinemic-euglycaemic clamp method Plus **any two of the following risk factors**: - Blood pressure ≥140/90mmHg or treatment - Triglycerides ≥1.7mmol/L or treatment - HDL-cholesterol <1.0mmol/L or treatment - BMI ≥30kg/m^2 or waist/hip ratio >0.85 - Albumin ≥20µg/min or albumin/creatinine ratio ≥30mg/g	**Fasting hyperinsulinemia** identified by: - Highest quartile of the non-diabetic population under study Plus **two or more of the following risk factors**: - Blood pressure ≥140/90mmHg or treatment - Triglycerides ≥2.0mmol/L or treatment - HDL-cholesterol <1.0mmol/L or treatment - Waist circumference ≥80cm - IFG (fasting glucose ≥6.1mmol/L)
NCEP-ATP III The National Cholesterol Education Program's Adult Treatment Panel III Report **2003**	**IDF criteria** International Diabetes Federation **2005**
Any three of the following risk factors: - Blood pressure ≥130/85mmHg or treatment Triglycerides ≥1.7mmol/L or treatment - HDL-cholesterol <1.1mmol/L or treatment - Waist circumference ≥88cm - IFG (fasting glucose ≥5.6mmol/L)	**Central obesity** - Waist circumference ≥80 cm or BMI≥30kg/m^2 Plus **two or more risk factors**: - Blood pressure ≥130/85mmHg or treatment - Triglycerides ≥1.7mmol/L or specific treatment for this lipid abnormality - HDL-cholesterol <1.3mmol/L or specific treatment for this lipid abnormality - IFG (fasting glucose ≥5.6mmol/L)

2.4.2 Prevalence of the metabolic syndrome

The prevalence of the MetS greatly depends on the definition used. However, the syndrome is estimated to increase with age has been increasing in the past decade. From NHANES in the US, reports of an age-adjusted prevalence of 24.1 % in 1988-94 was reported using the NCEP-ATP III-criteria. The prevalence increased with increasing age from 6.7% to 43.5% in the 20-29-year-old subjects compared to those aged 60-69 years, following a small decrease in elderly (103). In an investigation of eight European studies, the prevalence of the syndrome showed to increase with increasing age and it was more common in men than in women. In non-diabetic subjects, the prevalence of the syndrome, as defined by the WHO, was 7-36% for men and 5-22% for women in the ages 40-55 years, whereas application of the EGIR definition has reported a prevalence of 1-22% for men and 1-14% for women (104). An extensive review performed by Ford et al. in 2005 clearly describes how the estimates vary with whatever definition is applied; 6–7% for all-cause mortality, 12–17% for CVD, and 30–52% for T2DM. Ford underlines how further research is needed to establish the use of the MetS in predicting risk for death, CVD, and T2DM in various population subgroups (105).

2.5 Type 2 Diabetes Mellitus

The number of people with diabetes mellitus has more than doubled over the past three decades (106) and the disease is currently diagnosed in 366 million people worldwide (107). The fraction of affected people is projected to rise to 439 million by 2030 (108–110) representing 7.7% of the worldwide adult population aged 20-79 years, 90% of whom will be diagnosed with T2DM (110). In the industrialized world, the increase in T2DM prevalence is mainly due to population ageing (111) and increased prevalence of overweight and obese individuals (57,112). Further, global estimates of undiagnosed diabetes have been described by Beagley et al. to add another 174.8 million diabetics to the global estimate leaving 45.8% of all diabetes cases in adults to be undiagnosed (108). The prevalence of diabetes in Denmark is comparable to global estimates, with current projections of 380.000 (7%) of all Danish citizens having a registered diagnosis of diabetes. Another 200.000 are estimated to be undiagnosed and further 750.000 live with pre-diabetes (113).

The global epidemic of T2DM is closely related to increasing rates of overweight and obesity in all age groups (114) and as previously described, overweight is often accompanied by other risk factors, referred to as the MetS when occurring in combination (115). T2DM is a multifactorial disease with complex interactions between these risk factors. Specifically,

in elder people, higher contents of visceral fat have been reported to be the main determinant of IGT, following reduced insulin sensitivity ultimately resulting in T2DM. Increased pancreatic fat with declining beta-cell function has also been reported to play a major role in T2DM development (116).

The prevalence of T2DM increases progressively with age, peaking at 16.5% in men and 12.8% in women at age 75-84 years, and in Framingham Study subjects, diabetes or glucose intolerance was present in 30%-40% over the age of 65 (117). A significant increase in diabetes incidence has been observed especially within the elderly age group in Denmark. From 2000 to 2012, a 140% increase of physician-diagnosed diabetes within the 60-69 year age group, a 104% increase in the 70-79-year-olds, and an increase of 87% within the ≥80 years age group has been reported (118). Based on 2012 data from the Danish National Diabetes registry, physician-diagnosed diabetes is present in 155,480 Danish women of which 66% are aged ≥60 years (119). Danish female diabetics have a 40% relative increased mortality risk, based on the 2012 estimates, which is a decrease compared to the 83% increased risk reported in 1997. However, decreased risk of mortality does not imply decreased risk of morbidity and numbers from 2001 reveal that diabetes, and co-morbidities related to diabetes, costs 86 million DKK, daily (120).

Despite having the highest prevalence of diabetes of any age group, older individuals with multiple co-morbidities is not a well-studied population, as they are often excluded from randomized controlled trials of treatments. Further, the great heterogeneity of health status of older adults challenges the development of health strategies fitting this increasing population group (121).

2.6 Cardiovascular Disease

The broad description of CVD covers numerous problems, many of which are related to atherosclerosis with subsequent plaque formation (122,123). Among CVD, the diseases within the heart constitute around two-thirds of the cases, of which ischemic heart disease is the most prevalent type of CVD. Other types are apoplexia, heart failure, and arrhythmia (124). During the past decades, much knowledge has been achieved concerning the pathophysiology related to CVD with hypertension, smoking, hyperlipidaemia and diabetes being some of the abnormalities which are generally accepted as risk factors (125).

It is difficult to get exact numbers for the overall prevalence of CVD in the population. A person hospitalized or dying from CVD may have lived with the disease for many years

without contact with the healthcare sector. The Danish National Health survey (SUSY) from 2005 describe how a total of 305,000 Danish people were living with CVD, 17,500 died of CVD, and 86,000 were hospitalized with a total of 142,000 admissions (124). Further, a study based on consultations in general practice estimated that each year there are approximately 2.7 million contacts with general practice as a result of CVD in Denmark (126). CVD affects women to the same extent as men, but women get the disease at a higher age (124). Almost 40% of cardiovascular deaths are due to coronary heart disease, and around a quarter is due to apoplexia. This applies to both men and women, but women have relatively more deaths caused by diseases of the brain vessels and fewer deaths from ischemic heart disease than men. The same applies to admissions, where a third of cardiovascular admissions for women are due to CVD and two-thirds are caused by heart disease (124).

2.7 Excessive Tissue Degradation

There is an inexhaustible list of risk factors associated with the negative impact on healthy ageing. In the previous sections, some of the most well-characterized metabolic risk factors and following metabolic diseases were elucidated. The following section will focus on tissue degradation in relation to ageing.

The role of low-grade chronic inflammation is often described as a fundamental part of the ageing process (127). Also, tissue stiffening, which is a combination of increased collagen crosslinking through glycation, often occurring as by-products of lipid oxidation, contributes to the ageing process (128,129). The tissue stiffening of aged tissue makes it mechanically weaker and more rigid than young tissue (128,130). This changing mechanical state, combined with low-grade inflammation, can severely compromise the organisation of the extracellular matrix (ECM), which is the essential scaffold of all tissues, thereby modifying epithelial organization and function. This can potentially promote age-related fibro-proliferative diseases such as cancer, atherosclerosis, osteoarthritis, diabetic retinopathy etc. (131,132).

The common ground in all fibro-proliferative diseases is dysregulated tissue remodelling, leading to excessive and abnormal accumulation of extracellular matrix (ECM) components in the affected tissues (133–135). It is estimated that fibro-proliferative diseases account for 45% of all deaths in the developed world (133,136). The following sections will focus on fibro-proliferative diseases on a cellular level.

2.7.1 The extracellular matrix

The ECM is a three-dimensional protein structure providing support for tissues and regulating tissue homeostasis (137). The ECM turnover is tightly controlled during normal tissue homeostasis with old and damaged ECM proteins being degraded and replaced by new (138). The basic structure and composition of the ECM is similar across different tissues, however with variations in the ratio between different ECM proteins, specific protein isoform expression and post-translational modifications of the ECM molecules (138).

The ECM can be divided into the interstitial matrix and the basement membrane. The interstitial matrix primarily forms the connective tissue, whereas the basement membrane is a specialised layer of ECM dividing epithelial and endothelial cells from the underlying stroma (137). In the following sections, focus will solely be on collagen type I, which is the main component of the interstitial membrane of the ECM.

2.7.2 Collagen type I

Collagens are the most abundant proteins in the human body constituting 30% of the total protein mass. Currently, 28 types of collagens have been identified and grouped according to structure and function (139,140). Within the many types of collagen, collagen type I is the most abundant type expressed in most connective tissues being the major protein in bone, skin, tendon, ligaments, sclera, cornea and blood vessels where it assembles the extracellular space and provides tensile strength (138,140). Collagen type I is a fibrillar collagen, synthesized in the endoplasmatic reticulum as procollagen, most frequently composed of two $\alpha 1$ chains and one $\alpha 2$ chain. A key aspect of collagen type I is its posttranslational modifications, such as cleavage, cross-linking and degradation. These modifications are essential for correct synthesis and structural integrity of the collagen, but also for its tissue-specific functionality (140).

2.7.3 Collagen type I biomarkers

The great content of collagen type I combined with the constant turnover throughout the body, has resulted in specified measurements of various fragments of this specific biomarker in blood samples (140,141). Biomarkers related to collagen type I are divided into two categories; formation and degradation markers.

The formation markers describe released pro-peptide fragments of collagen type I, which are cleaved off during the synthesis of the collagen triple-helix. The amino-(N)-terminal pro-peptide of procollagen type I is termed PINP, whereas PICP refers to the

carboxy-(C)-terminal pro-peptide. Both formation markers are described in studies where they are used to reflect the synthesis of bone matrix, and PINP is often used in drug trials assessing bone formation and turnover (142,143).

Collagen type I degradation markers are divided into two types; cathepsin K-generated collagen type I degradation fragments and matrix-metalloproteinase (MMP)-mediated collagen type I degradation fragments. The most well-described degradation marker is the C-terminal telopeptide of collagen type I (CTX-I). CTX-I reflect bone resorption and has been used extensively in clinical studies of anti-resorptive drugs treating osteoporosis (142,143).

2.7.4 C1M – a novel biomarker for collagen type I degradation

C1M is categorised as an MMP-mediated collagen type I degradation fragment. This fragment is not related to bone turnover as it is not released as a function of bone resorption (144). Studies have shown that C1M is closely related to chronic inflammation and therefore has potential as a biomarker across multiple diseases, including rheumatoid arthritis, osteoarthritis, and fibro-proliferative diseases (145–147). These initial findings on associations between serum levels of C1M and subsequent disease activity, support the assumption, that MMP-mediated destruction of collagen type I is a pathologically relevant process associated with fibro-proliferative diseases, rather than bone formation, and that monitoring these specific fragments of collagen type I can provide clinical value in relation to healthy ageing.

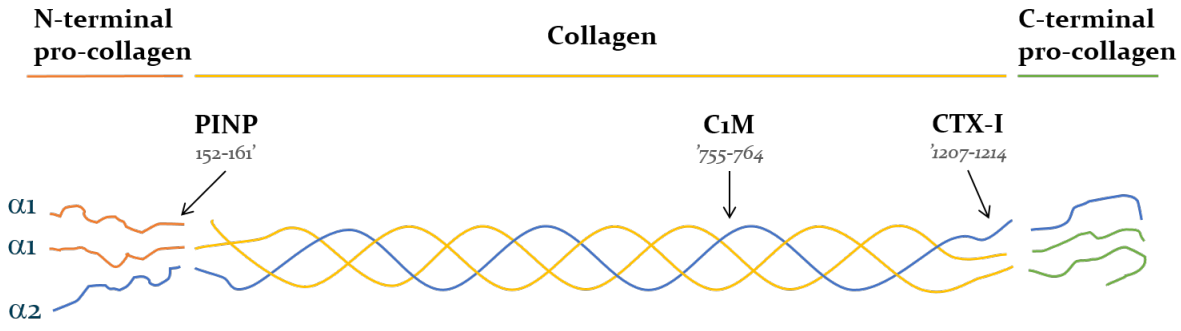

Figure 2 Location of collagen type I biomarker target sites, modified with permission from Siebuhr et al (148).

2.7.5 Measuring disease burden

From above description of formation and degradation fragments of collagen type I, it is clear, that measurements of the same protein can contain distinct information and reflect completely different biological processes depending on which part of the protein is targeted. Identifying and assessing different post-translational modifications, such as pro-peptides or degradation products, provides a distinct 'protein fingerprint' which thereby makes it possible to distinguish different pathophysiological processes. Described in other terms; whereas the total pool of collagen type I may not change during pathological conditions, the various 'protein fingerprints' may differ significantly. In these cases, the quantification of a protein sub-pool may more accurately describe the pathology of interest, e.g. in elderly women, where bone resorption can be extensive in late-life, knowledge on increased CTX-I levels are of greater interest than quantifying full-length collagen type I.

2.7.6 The protein fingerprint technology – measuring C1M in serum

The fingerprint approach is founded on the previously described principals that specific protein fragments are released into the circulation where they can be measured. Proteases degrade proteins by cleaving them at a specific amino acid sequence and hereby expose a protease-specific epitope (neo-epitope) on the degraded protein fragment that can be targeted as a biomarker.

The protein fingerprint technology is based on competitive enzyme-linked immune-sorbent assay (ELISA) tests, using monoclonal antibodies to target the specific protease-generated neo-epitopes. In the case of C1M, this would be the specific MMP-mediated fragment generated during tissue turnover. During pathological process, this fragment would be assumed to be up-regulated.

The development of the C1M assay has been performed by colleagues at Nordic Bioscience and is fully described in the paper by Leeming et al. (144). In this thesis, further emphasis will not be put upon the technical aspects of C1M measurement but solely on its use as a descriptive biomarker of increased risk of age-related diseases and consequent death in elderly women.

2.8 Introductory Overview

The main focal points of the introduction are summed up in Figure 3, thus giving rise to the specific aims formulated in Chapter 3 forming the basis of the presented work.

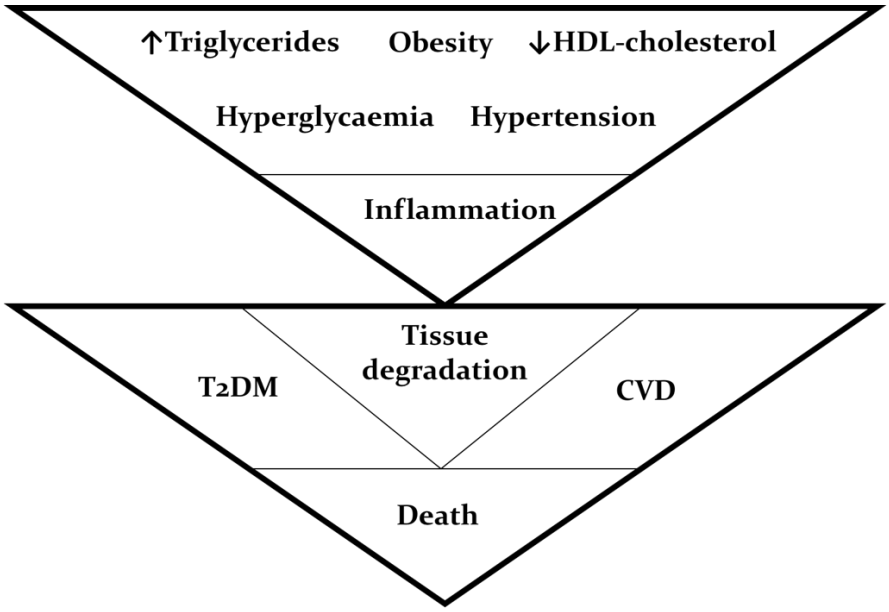

Figure 3 Risk factors of the metabolic syndrome combined with the low-grade inflammation related to ageing lead to tissue degradation following adverse metabolic outcomes such as type 2 diabetes (T2DM) and cardiovascular diseases (CVD) and ultimately death.

3 AIM

There is a need to specifically investigate healthy ageing in elderly women. Ageing is a broad term describing the accumulation of various changes over time. In the broadest sense, it can refer to everything from cellular ageing of an organism to the ageing of populations encompassing both physical, psychological and social changes. The studies in the current thesis focus solely on ageing in an epidemiological population-based setting describing risk factors directly linked to physical ageing and associated non-communicable diseases.

The overall aim of this thesis was to study risk factors affecting healthy ageing in women. The Prospective Epidemiological Risk Factor Study (PERF), a community-based cohort comprising Danish elderly women was the focal point of the conducted research. The cohort is described in detail in the form of a cohort profile (study I). The specific aims of the three studies that found the basis of the current work all relate to the interplay between risk factors and age-related non-communicable diseases.

The specific aims were to:

1. Investigate the predictive value of the MetS definition in relation to future risk of T2DM and CVD by applying the MetS definition set by the IDF. Further, the aim was to investigate whether the syndrome's predictive power of T2DM and CVD would increase by further stratifying the reference group. Lastly, the aim was to investigate the risk of T2DM and CVD based on cumulating numbers of MetS risk factors (study II).

2. Investigate the influence of weight and weight change during a period of 13-years on the subsequent risk of hyperglycaemia development specifically exploring the risk within normalweight, overweight and obese women (study III).

3. Investigate whether MMP-mediated tissue degradation of collagen type I could predict increased risk of premature mortality in elderly women (study IV).

4 INTRODUCTION TO THE COHORT

Title

Cohort Profile: The Prospective Epidemiological Risk Factor (PERF) study

Aim

The PERF study is an ambi-directional population-based study of postmenopausal women set up with the purpose of obtaining a better understanding of the aetiology and pathogenesis of age-related diseases.

Rationale

The world's population is ageing. Knowledge of disease and health in this fast growing segment of the population is largely built upon population-based studies of the distribution and determinants of disease outcomes. The PERF study was designed with the purpose of obtaining a better understanding of the development of age-related diseases in postmenopausal women.

Findings

The cohort profile outlines the study design, the study population, and an overview of the collected data together with a summary of the key findings until now.

The average lifespan in the cohort was found to be very similar to the average lifespan for Danish women. When compared to Danish women aged 45+ the PERF cohort is characterized as slightly less physically active and more overweight/obese. The number of current smokers is less in the cohort while the group of subject's not drinking alcohol is larger in our cohort compared to Danish women aged 45+. In relation to health, the two main causes of death are CVD and cancer in both the cohort and in the background population, and the proportion of deaths attributable to these diseases are comparable. For other comorbidities, the proportion of subjects with diabetes and depression in the cohort are similar to the target population, while the prevalence of hypertension and osteoporosis is approximately 2-fold higher in the cohort.

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Cohort Profile

Cohort Profile

Cohort Profile: The Prospective Epidemiological Risk Factor (PERF) study

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Why was the cohort set up?

The world's population is ageing.[1] In Europe alone, the elderly population over age 65 will double from 88 to 153 million and the fastest growing segment of the population will be those over 80, tripling in number from 24 to 60 million in 2060.[2] Low birth rates and increasing longevity are the key factors in this shifting trend in ageing demographics.[3] Maintaining a healthy life is important, as an ageing population in good health will limit the pressure on health care systems.[3,4] However, it is likely that risk factors compromising healthy ageing, such as smoking, obesity, excess alcohol consumption, unemployment, and lack of physical activity, will negatively affect the years people spend in good non-treatment requiring health.[1,5] In 2006, it was estimated that women in the Western European countries are expected to live about 80% of their lives in good health. In other words, this predicts a healthy life expectancy up to 20% shorter than the total life expectancy.[4] Focus on a healthy elderly population is therefore of greater interest than ever.

Age-related diseases are usually expressed as chronic conditions commonly occurring in combination with each other, with cardiovascular disease and type 2 diabetes being two of the most common age-related diseases in the EU.[1,4] The ability to understand the links and underlying pathogenesis are therefore crucial in order to be able to shift the treatment regimen from disease treatment to preventive measures, thereby prolonging the period that elderly people spend in good health.

The Prospective Epidemiological Risk Factor (PERF) Study, an observational, prospective cohort study of Danish postmenopausal women, was designed with the purpose of obtaining a better understanding of the development of age-related diseases in postmenopausal women. In 1999, the source population was identified from a database of subjects who had previously been screened for participation in one of 21 clinical randomized controlled trials (source studies[6–24]). All living subjects with a unique personal subject identification number and a valid postal address constituted the source population (a total of 8875 women). The source studies were all initiated with the purpose of obtaining further knowledge about the aetiology and pathogenesis of menopause-related diseases, and included both intervention and non-intervention studies (as illustrated in Figure 1). The source population therefore consists of women who previously participated in a source study or were screened, without being randomized. The first source study was initiated in 1977. In 1999, the first participants were enrolled in the epidemiological cohort of the PERF study (henceforth termed PERF I), and from September 2013 to December 2014 the participants completed the latest follow-up (termed PERF II). The total number of participants attending the baseline examination

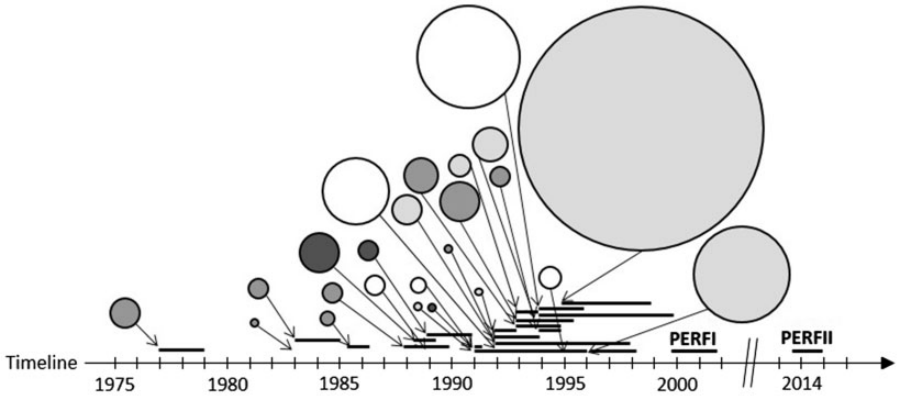

Figure 1. Source studies defining the source population for the Prospective Epidemiological Risk Factor (PERF) study illustrated on a timeline including number of participants, study duration and type of intervention. Bubble size is proportional and equivalent to the number of participants. All bubbles are sized relative to the largest study (N = 2,789). Color represents type of intervention; white bubbles are nonintervention studies, light grey bubbles are placebo controlled bisphosphonate studies, medium grey bubbles are placebo controlled hormone replacement therapy studies and dark grey bubbles represent other types of intervention studies. Black lines correspond to the study duration (in years).

(PERF I) was 5855, of whom 2103 attended the follow-up visit (PERF II) approximately 15 years later. Including the source studies, the study may be considered an ambidirectional cohort study with a total observation period of more than 35 years. The PERF I and PERF II studies were funded by the Danish Research Foundation (Den Danske Forskningsfond).

The current paper outlines the study design, the study population and an overview of the collected data together with a summary of the key findings until now.

Who is in the cohort?

Inclusion

In 1999, an invitation to attend the baseline examination was sent to the entire source population ($n = 8875$) except for those who died since their last contact with the clinic ($n = 732$). In this subgroup, causes and times of death were collected from the Danish National Death registry. No active recruitment initiatives besides the invitation was taken, leaving a total of 5855 (72%) women to consent and attend the baseline examination of the epidemiological PERF I study conducted at the Center for Clinical and Basic Research (CCBR) in cities of either Aalborg or Ballerup, Denmark, between 1999 and 2001. There were no in-/exclusion criteria at the time of enrolment in the cohort study.

A subcohort (PERF II), initially being enrolled at the CCBR clinic in Ballerup, was re-investigated in 2013-14, when invitations were sent to 2813 women from the original PERF I cohort. Those subjects who did not respond to the written invitation were contacted by phone. As a result of this active recruitment, a total of 2103 (75%)

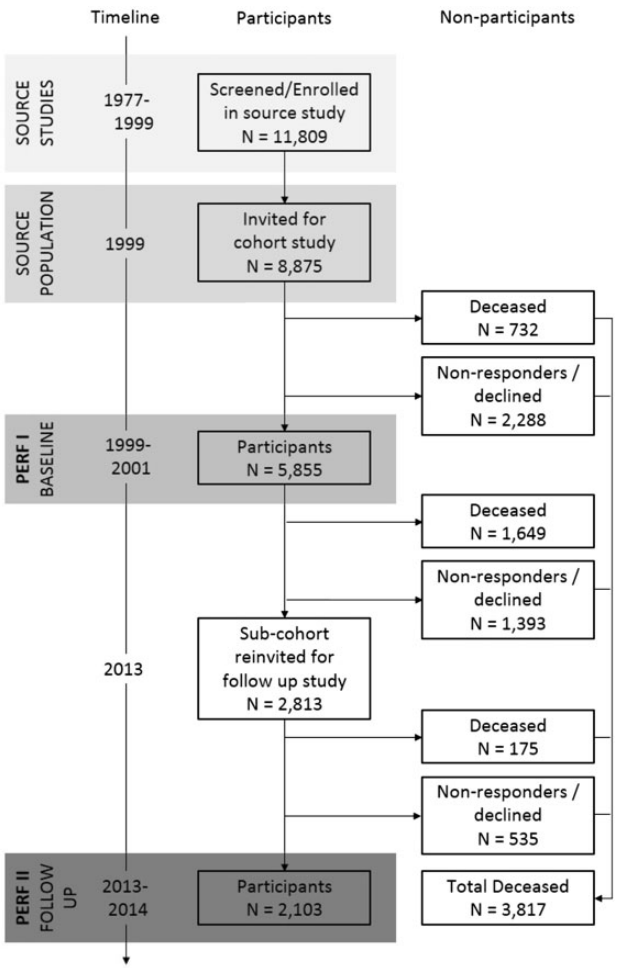

Figure 2. Flowchart of participants and >non-participants in the baseline and the follow-up study. The repeating occurrence of deceased and non-responders/declined illustrate the number of deceased and nonresponders/declined between two consecutive time points on the time scale.

Table 1. Selected baseline characteristics of the Prospective Epidemiological Risk Factor (PERF) study. The full study population ($n = 5855$) are shown along with specific subgroups of; subjects who died before follow-up ($n = 1649$), subjects who attended the follow-up visit (PERFII) (n = 2103) and subjects who did not attend the follow-up visit ($n = 2103$). Numbers are shown as absolute numbers with percentile in brackets for categorical variables. For numerical variables, the mean ± standard deviation (SD) are shown

Parameter	*N*	Baseline Participants (PERF I) *N* = 5855	Dead before follow-up *N* = 1649	Follow-up participants (PERF II) *N* = 2103	Follow-up non-participants *N* = 2103	***P*-value* Follow-up participants vs non- participants**
Age (mean ± SD, years)	5855	70.8 (6.5)	74.9 (5.9)	68.0 (6.0)	70.3 (5.9)	<0.001
Menopause age (mean ± SD, years)	5783	49.0 (4.9)	48.7 (5.0)	49.1 (4.8)	49.1 (4.8)	0.9
Highest level of education	5841					<0.0001
Primary school, *n* (%)		4178 (72)	1215 (74)	1428 (68)	1535 (73)	
High School, *n* (%)		1250 (21)	320 (20)	482 (23)	448 (21)	
University, *n* (%)		413 (7)	110 (7)	192 (9)	111 (5)	
Height (mean ± SD, cm)	5637	161 (5.9)	160 (6.0)	162 (5.8)	161 (5.8)	<0.001
Weight (mean ± SD, kg)	5637	67.7 (11.7)	65.5 (11.9)	68.8 (11.4)	68.4 (11.6)	0.2
BMI (mean ± SD, kg/m^2)	5637	26.1 (4.3)	25.7 (4.4)	26.2 (4.2)	26.5 (4.2)	0.07
BMI groups	5637					0.1
Underweight (<18.5), *n* (%)		90 (2)	46 (3)	21 (1)	23 (1)	
Normal (≥ 18.5-25.0), *n* (%)		2343 (42)	699 (45)	871 (42)	773 (38)	
Overweight (> 25.0-30.0), *n* (%)		2248 (40)	567 (37)	823 (40)	858 (43)	
Obese (> 30.0), *n* (%)		956 (17)	238 (15)	356 (17)	362 (18)	
Smoking	5844					0.6
Never, *n* (%)		2767 (47)	634 (39)	1077 (51)	1056 (50)	
Past, *n* (%)		1762 (30)	525 (32)	610 (29)	627 (30)	
Current, *n* (%)		1315 (23)	487 (30)	416 (20)	412 (20)	
Alcohol	5807					<0.0001
Never, n (%)		2531 (44)	757 (46)	843 (40)	931 (45)	
<10.5 alcohol units/week, n (%)		1380 (24)	348 (21)	451 (22)	581 (28)	
10.5-21 alcohol units/week, n (%)		1497 (26)	423 (26)	615 (29)	459 (22)	
>21 alcohol units/week, n (%)		399 (7)	107 (7)	180 (9)	112 (5)	
Physical activity	5843					0.05
Never, *n* (%)		1840 (31)	720 (44)	525 (25)	595 (28)	
1 time/week, *n* (%)		1233 (21)	340 (21)	451 (21)	442 (21)	
2 times/week, *n* (%)		748 (13)	179(11)	308 (15)	261 (13)	
3+ times/week, *n* (%)		2022 (35)	408 (25)	819 (39)	795 (38)	
Blood pressure						
Systolic (mean ± SD, mmHg)	5677	150 (24.4)	155 (25.4)	147 (23.3)	150 (24.2)	<0.001
Diastolic (mean ± SD, mmHg)	5679	81.9 (11.5)	81.7 (12.3)	82.0 (10.7)	81.8 (11.6)	0.6
Hypertension, *n* (%)	5838	1807 (31)	606 (37)	523 (25)	678 (32)	<0.0001
Hyperlipidaemia, *n* (%)	5845	530 (9)	142 (9)	224 (11)	164 (8)	0.002
Diabetes, *n* (%)	5842	181 (3)	75 (5)	47 (2)	59 (3)	0.06

*t test for numerical variables and chi-square test for categorical variables.

women attended the follow-up study (PERF II), which took place either in their own home or at the CCBR clinic in Ballerup. Figure 2 shows the number of participants and non-participants from baseline to follow-up. All the subjects were given ample time to consider participation and gave their written consent before any study-related procedure was carried out. The study was conducted in accordance with Good Clinical Practice and the Helsinki Declaration II.

Cohort characteristics, a comparison between baseline participants, follow-up participants and non-participants

The baseline characteristics of the entire cohort (PERF I) and the follow-up participants (PERF II) are shown in Table 1. The mean age in the baseline cohort (PERF I) was 70.8 years (49.7-88.8). Nearly 75% of the cohort had primary school as their highest level of education and less than 10% had a university degree. The follow-up

participants were characterized as being younger and slightly higher-educated. With an average BMI of 26.2 kg/m^2, this part of the cohort comprised 57% overweight or obese women. There were no differences between the follow-up participants and non-participants with regards to BMI. In relation to lifestyle variables (smoking, alcohol and physical activity), follow-up participants and non-participants for PERF II were found to be similar, although the follow-up participants comprised a higher proportion of subjects consuming > 10.5 alcohol units per week. The systolic blood pressure and the proportion of subjects with self-reported hypertension were higher in the group of non-participants than in the participating group, whereas the proportion of subjects with self-reported hyperlipidaemia was lower.

Cohort and target population characteristics

Comparison of study participants with the target population was done using data on Danish women aged 45+, from the Danish Health Interview Surveys (SUSY) in 2000[25] and 2005[26] and the StatBank from Statistics Denmark[27] (Table 2).

The average lifespan in the cohort is very similar to the average life span for Danish women. When compared with Danish women aged 45+ generally, the PERF cohort is characterized as slightly less physically active and more overweight/obese. The proportion of current smokers is less in the cohort and subjects not drinking alcohol is larger in our cohort compared with Danish women aged 45+. In relation to health, the two main causes of death are cardiovascular disease and cancer in both the cohort and the background population, and the proportions of deaths attributable to these diseases are comparable. For other comorbidities, the proportions of subjects with diabetes and depression in the cohort are similar to the target population, but the prevalences of hypertension and osteoporosis are approximately 2-fold higher in the cohort.

Table 2. Comparison of the PERF cohort and the target population comprising Danish women aged 45 and older. Data on the target population are derived from either Statistics Denmark or the Danish Health Interview Surveys. Values are shown as percentages if not otherwise indicated

Variable	Baseline cohort (PERF I)	Danish Women 45 + (target population)	*P*-value[e]
Demography and lifestyle			
Age (% of total group)			
60-64	18.3	25.3[a]	<0.01
65-69	23.2	22.0[a]	0.02
70-74	28.7	20.4[a]	<0.01
75-79	20.6	18.9[a]	<0.01
80-84	9.2	13.4[a]	<0.01
Average lifespan (years)[b]	83.0	82.7[a]	
Smoking (% of total group)			
Current	22.5	31.9[‡]	<0.01
Never	47.3	39.8[‡]	<0.01
Alcohol (% of total group)			
Never	43.6	28.2[c]	<0.01
<10.5 alcohol units/week	23.8	44.1[c]	<0.01
10.5-21 alcohol units/week	25.8	18.2[c]	<0.01
> 21 alcohol units/week	6.9	9.5[c]	<0.01
Physical activity (% of total group)			
No	31.5	21.9[c]	<0.01
Yes	68.5	78.1[‡]	<0.01
BMI (% of total group)			
Underweight (<18.5)	1.6	4.1[c]	<0.01
Normal weight (≥ 18.5 <25)	41.6	54.4[c]	<0.01
Overweight (≥ 25)	39.8	30.8[c]	<0.01
Obese (≥ 30)	17.0	10.7[c]	<0.01
Health			
Causes of death (% of total group)			
Cardiovascular	27.3	25.7[a]	
Cancer	32.2	33.8[a]	
Comorbidities (% of total group)			
Hypertension	31.0	16.4[c]	<0.01
Diabetes	3.1	3.9[c]	0.02
Osteoporosis	10.9	6.1[d]	<0.01
Depression/anxiety	6.6	5.5[d]	0.02

[a]Retrieved from Statistics Denmark.
[b]The average lifespan was calculated for all deceased subjects by the end of 2014.
[c]Data from the Danish Health Interview Surveys 2000.
[d]Data from the Danish Health Interview Surveys 2005.
[e]The z-score test for two population proportions.

How often have they been followed up?

Concomitant with the PERF II follow-up study, all subjects have been followed with registry linkage using population-based national registries. With approval from the authorities, we have collected registry data on all baseline participants ($n = 5855$). By use of a personal subject identification number (CPR-number), the Danish national registries contain individual-level data on the entire Danish population. Linkage has been done with the following registries: the National Danish Patient Registry, the National Danish Causes of Death Registry, the Danish National Diabetes Register, the Danish Cancer Registry and the Danish National Pathology Registry. For more information on the registries, please refer to Table 3.

The most recent linkage was done in January 2015, and this linkage is expected to continue until the remaining subjects from the cohort are deceased. The registry information is available for research within the scope of the study.

Table 3. Overview of registry linkage in the Prospective Epidemiological Risk Factor (PERF) study

Registry	Type of information received	Time period covered	Latest linking
National Danish Patient Registry	Hospitalization and discharge time	1977 on	31 Dec 2014
	Hospital and department	1977 on	
	Diagnoses (ICD classification)	1977 on	
	Treatments and operations	1996 on	
National Danish Causes of Death Registry	Time of death	1970 on	31 Jan 2015
	Underlying cause of death	1970 on	
	Complementary cause of death	1970 on	
Danish National Diabetes Register	Date of inclusion	1990 on	31 Dec 2014
	Inclusion criteria	1990 on	
	Inclusion cause	1990 on	
Danish Cancer Registry	Diagnosis and time of diagnosis	1943 on	31 Dec 014
	Tumour distribution	1943-2003	
	Treatment	1943-2003	
	TNM classification	2004 on	
	Ann Arbor staging	2004 on	
	Treatment	1943-2003	
Danish National Pathology Registry	Data from pathological tests (by SNOMED code)	1997 on (1970 on)	31 Dec 2014

What has been measured?

Baseline and follow-up examination

At the baseline visit (PERF I), participants completed a health examination involving a physical examination including blood pressure measurement, electrocardiogram (ECG), medical history and a health-related questionnaire (for more information on the questionnaire see separate section below). Participants provided blood and urine samples for standard biochemical analysis and for future analysis by storage in a biobank. Moreover, dual energy X-ray absorptiometry (DEXA) scans of the whole-body, spine, hip and arm, X-ray of the spine and mammography were obtained.

At the follow-up examination (PERF II), medical history and recording of all current medications were obtained. Measurements of height, weight, waist and hip circumferences, blood pressure, heart rate and respiratory frequency was completed. Muscle strength was determined using a hydraulic hand-grip dynamometer. An EQ-5D-3L evaluation was completed by the participant to assess their self-reported quality of life, and a Category Fluency Test together with a Short Blessed Test was done to test cognitive performance. Please refer to Table 4 for information on the data collected at the baseline and the follow-up examination.

Questionnaire

The baseline and follow-up questionnaire was completed as a structured interview with an investigator or study nurse and the participant. Standard demographic information such as age, menopause age and level of education, along with information on physical activity, current and past smoking habits and current and past drinking behaviour was included in the questionnaire. Information on diet obtained at baseline was limited to information on consumption of coffee/tea, dairy products and vegetarian status. Medical history, including treatment (medication/surgical) and familial medical history, was obtained as part of the interview for several disorders including, but not limited to, neurological or psychological disorders, cardio-/cerebrovascular disease, lung disorders, cancers, muscles and joint diseases and metabolic disorders.

Collection, analysis and storage of biological material

For each participant, urine and fasting blood samples were collected for routine analysis and biobank storage at baseline ($n = 5668$). The biobank also contains DNA samples for those subjects who gave written consent for this specific analysis ($n = 5553$). At the follow-up visit, fasting blood samples were collected. Samples are stored at -20 °C (urine, DNA samples) and -80 °C (serum). Routine blood and urine analysis was carried out at a College of American Pathology (CAP) certified central laboratory (Nordic Bioscience Laboratory) at both baseline and follow-up.

Genomics

In collaboration with deCODE genetics, Iceland, and Sct. Hans Hospital, Denmark, DNA samples from the PERF study have been genotyped and associations between single nucleotide polymorphisms (SNPs) and selected outcomes, including bone mineral density/osteoporotic fractures,[34]

Table 4. Parameters measured at the baseline (PERF I) and the follow-up visit (PERF II)

Parameter	Description	PERF I	PERF II
General information			
Demographics	Age	√	NA
	Body weight	√	√
	Height	√	√
	Education level	√	NA
Health			
Medical history	Self-reported questionnaire/interview	√	√
Physical examination	Full-body examination	√	—
	Blood pressure	√	√
	ECG	√	—
Cognition	Short Blessed Test	√	√
	Category Fluency Test (Animals)	√	√
Body composition	Arm, hip and spine DEXA	√	—
	Whole-body DEXA	√	—
X-ray	Spine	√	—
	Mammography	√	—
Muscle strength	Hand-grip strength test	—	√
Lifestyle			
Physical activity	Walking, leisure activity	√	√
Smoking	Current and past smoking behaviour	√	√
Alcohol	Current and past drinking behaviour	√	√
Diet	Consumption of coffee/tea, dairy products	√	—
	Vegetarians	√	—
Psychosocial parameters			
Quality of life, well-being	EQ-5D-3L[a]	—	√
Blood			
Haematology	Haemoglobin, leukocytes and differentiation, etc.	√	√
Lipids	Total cholesterol, LDL, HDL, triglycerides	√	√
Electrolytes	Sodium, potassium, calcium	√	√
Renal function	Creatinine	√	√
Liver	ALAT, ASAT, albumin, GGT, alkaline phosphatase	√	√
Inflammation	High sensitive CRP	—	√
Specialty biomarkers	Osteocalcin, CTX-1, VICM, C1M, C4M, TAU-C	√	*

NA, not applicable.

[a]EQ-5D-3L measures health in five dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression) and three levels (no problems, some problems, extreme problems).

type 2 diabetes,[35] schizophrenia,[36] depression[37] and cognitive impairment[38] have been assessed.

What has it found? Key findings and publications

The PERF study has generated several important findings covering the health of elderly women. Selected key findings are summarized in Table 5. In a cross-sectional nested analysis from PERF ($n = 1356$), it was shown that peripheral adiposity exhibits an independent anti-atherogenic effect in elderly women.[39,40] In the entire cohort and in a nested study ($n = 343$), it was shown that endogenous estrogen and hormone replacement therapy administered in the early phase of the menopause may have a protective association with cognitive impairment later in life.[41,42] More recently, it was shown that matrix metalloproteinase (MMP)-mediated collagen type I degradation, termed C1M, is an independent risk factor for all-cause mortality, as subjects with high levels of type I collagen degradation had a 2-fold increased mortality risk compared with subjects with low levels.[43] Last, a genome-wide association study of bone mineral density (BMD) among more than 30 000 subjects, including samples from PERF I, revealed a new BMD locus that harbours the PTCH1 gene. The gene is associated with reduced spine BMD.[44]

What are the main strengths and weaknesses?

In this 37-year ambidirectional population-based study, the participation rate has been higher than 70% throughout the study. To investigate whether the study population

Table 5. Summary of major findings from the Prospective Epidemiological Risk Factor (PERF) study

Endpoint/Exposure	Major findings
Cardiovascular disease	Localization of fat mass is more important for atherogenesis than obesity per se[39,40]
	Enlarged waist circumference and elevated triglycerides are simple diagnostic tools that could facilitate the identification of postmenopausal women at increased risk for accelerated atherogenesis and related adverse outcomes[45]
	Hormone replacement therapy for 2-3 years has relative cardiovascular benefits and reduces the risk of all-cause mortality[46]
Bone/osteoporosis	Limited hormone replacement therapy given in the early postmenopausal years can provide long-lasting benefits in terms of preventing bone loss and related fractures[47]
	Bone mass measurement offers effective fracture prediction independent of the site of measurement and age of the patient[48]
Association of conditions	Aortic calcification seems to independently contribute to the development of osteoporosis in the proximal femur[49]
	Independent association of peripheral vascular disease with osteoporosis in the proximal femur[50]
Cognitive function	Protective association of body fat mass with cognitive impairment in elderly women, through a more prominent exposure to endogenous estrogens[41]
	Short-term hormone replacement therapy administered in the early phase of the menopause may provide a long-term protection against cognitive impairment[42]
Genomics	Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes[35]
	Association of dopamine beta-hydroxylase gene variants with cognitive performance and depression in elderly women[37,38]
	Bone mineral density locus identified that harbours the PTCH1 gene. The gene is associated with reduced spine BMD[44]
All-cause mortality	Increased MMP-mediated tissue degradation, measured by C1M, is an independent risk factor for all-cause mortality[43]

resembled the target population, we compared the baseline participants with the general female population in Denmark aged 45 or older. It is well known that study participation is often linked to health superior to that of an otherwise similar, non-participant background population (healthy participant bias). However, we did not observe a healthier profile among the baseline study participants. The cohort is therefore considered representative of women aged 45+ in the Danish population. In the study population, we found a higher prevalence of osteoporosis at baseline. This could either be caused by selection bias, as a number of the participants had previously participated in randomized clinical trials focused on osteoporosis. However it could also reflect underdiagnosis of osteoporosis in the general Danish population, since the source population not only included women randomized for clinical studies but also those who did not meet the inclusion criteria (e.g. had high bone mineral density) at the time of recruitment.

Although the follow-up cohort (PERF II) was selected based on geographical limitations due to data collection in the participants' own homes, the similarities between follow-up participants and follow-up non-participants strengthen the internal validity of the data.

Besides the length of the follow-up period, the linkage to a range of nationwide registries is a major strength. The registry data are comprehensive and the registries were established relatively early, e.g. cancer and cause-specific death information since the 1940s and hospitalizations since the 1970s.[51] Registration has been mandatory since 1977. The registry data therefore strongly support the identification of outcomes and, because of the limited loss to follow-up, it adds analytical power to the study. Moreover, this cohort is to our knowledge one of the largest cohorts of postmenopausal women with full-body DEXA scans, which enables extensive studies of body composition.

Regarding weaknesses, the cohort only comprised women and therefore generalization cannot be made to men of similar ages. Moreover, the duration of time passed from PERF I (year 1999) to PERF II (year 2014) is long in a cohort of such advanced age. In order to prevent selection bias towards the healthier segment of this ageing cohort, great effort was made in following up on invited participants not instantly replying to our invitation. Also, visiting the subjects in their own homes increased the number of subjects with illnesses still wanting to participate.

Can I get hold of the data? Where can I find out more?

All data are stored electronically in anonymous form. Aliquoted biological material is stored in a biobank at the Nordic Bioscience Laboratory. Currently, the data are available only to employees of Nordic Bioscience A/S, Denmark; however, the PERF study group will welcome any enquiries regarding collaboration or data sharing for further investigations. Potential collaborators are invited to contact the PERF study group at [perf@nordicbio.com].

Profile in a nutshell

- The Prospective Epidemiological Risk Factor (PERF) Study is an ambidirectional population-based study of postmenopausal women set up with the purpose of obtaining a better understanding of the aetiology and pathogenesis of age-related diseases.
- Participants were recruited from a source population of 8875 women residing in Denmark. The baseline examination (PERF I) comprised 5855 women with mean age of 70.8 years (49.7-88.8) and took place between 1999 and 2001.
- All subjects have been followed up with registry linkage using population-based national registries. Further, a subcohort was re-invited to attend a follow-up visit between 2013 and 2014 (PERF II). Registry data are available for all baseline participants. From the baseline population, 2103 were enrolled in PERF II.
- The data repository contains a wide range of health-related and lifestyle measures, biological samples from the baseline and follow-up studies, genetic information and linkage to nationwide registries.
- The PERF study group will welcome any enquiries regarding collaboration or data sharing for further investigations.

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Conflict of interest: C.C. and B.J.R. serve as board members and stock owners in Nordic Bioscience A/S. M.A.K., J.R.A., P.Q. and A.B. hold stocks in Nordic Bioscience A/S.

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5 METABOLIC SYNDROME IN ELDERLY WOMEN

Title

Metabolic Syndrome and Subsequent Risk of Type 2 Diabetes and Cardiovascular Disease in Elderly Women: Challenging the Current Definition.

Aim

The aim of the study was to investigate the association of risk factors of the metabolic MetS with later risk of T2DM and CVD in elderly Caucasian women. We further investigated if stratification of individuals not defined with MetS would add predictive power in defining future disease in individuals with MetS.

Rationale

The prognostic value of the MetS is believed to vary with age. With an elderly population expecting to triple by 2060, it is relevant to evaluate the validity of defining the MetS in this age group.

Findings

Elderly women with defined MetS presented a 6.3-fold increased risk of T2DM (95% CI: [3.7-10.5]) and 1.7-fold increased risk of CVD ([1.4-2.1]) compared to women with no MetS risk factors. Sub-dividing the control group without defined MetS revealed that both centrally obese controls and controls holding other MetS risk factors also had an increased risk of T2DM (HR=2.2 [1.3-3.9] and HR=1.8 [1.0-3.0], respectively) and CVD (HR=1.5 [1.3-1.8] and HR=1.4 [1.2-1.6], respectively) when compared to controls with no MetS risk factors. The risk of both T2DM and CVD increased with cumulative numbers of metabolic risk factors.

Conclusions

MetS in elderly Caucasian women increased the risk of future T2DM and CVD. While not defined with MetS women holding only some risk factors for MetS were also at increased risk of T2DM or CVD compared to women with no MetS risk factors.

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Metabolic syndrome and subsequent risk of type 2 diabetes and cardiovascular disease in elderly women

Challenging the current definition

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Abstract

The prognostic value of the metabolic syndrome (MetS) is believed to vary with age. With an elderly population expecting to triple by 2060, it is important to evaluate the validity of MetS in this age group. We examined the association of MetS risk factors with later risk of type 2 diabetes (T2DM) and cardiovascular disease (CVD) in elderly Caucasian women. We further investigated if stratification of individuals not defined with MetS would add predictive power in defining future disease prevalence of individuals with MetS.

The Prospective Epidemiological Risk Factor Study, a community-based cohort study, followed 3905 Danish women since 2000 (age: 70.1 ± 6.5) with no previous diagnosis of T2DM or CVD, holding all measurements used for MetS definition; central obesity, hypertension, hyperlipidemia, and hyperglycemia combined with register-based follow-up information.

Elderly women with defined MetS presented a 6.3-fold increased risk of T2DM (95% confidence interval: [3.74–10.50]) and 1.7-fold increased risk of CVD (1.44–2.05) compared to women with no MetS risk factors. Subdividing the control group without defined MetS revealed that both centrally obese controls and controls holding other MetS risk factors also had increased risk of T2DM (hazard ratio (HR) = 2.21 [1.25–3.93] and HR = 1.75 [1.04–2.96]) and CVD (HR = 1.51 [1.25–1.83] and HR = 1.36 [1.15–1.60]) when compared to controls with no MetS risk factors.

MetS in elderly Caucasian women increased risk of future T2DM and CVD. While not defined with MetS, women holding only some risk factors for MetS were also at increased risk of T2DM or CVD compared to women with no MetS risk factors.

Abbreviations: ALAT = alanine-aminotransferase, ASAT = aspartate-aminotransferase, BMI = body mass index, C/P ratio = central/peripheral fat mass ratio, CCBR = Center for Clinical and Basic Research, CVD = cardiovascular disease, DEXA = dual-energy X-ray absorption, HDL = high-density lipoprotein, IDF = International Diabetes Federation, LDL = low-density lipoprotein, MetS = metabolic syndrome, PCA = principal component analysis, PERF = Prospective Epidemiological Risk Factor Study, T2DM = type 2 diabetes mellitus, WBC = white blood cell count.

Keywords: cardiovascular disease, central obesity, elderly, metabolic syndrome, principal component analysis, type 2 diabetes

1. Introduction

The risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) increases with age,[1–3] and with a generally aging population,[4] definite measures of disease risk in elderly individuals are necessary. Such strategy would facilitate timely preventive approaches to reduce the disease burden, as well as medical costs in an aging population.[5,6] Metabolic syndrome (MetS) is widely used as a measure to predict the future risk of T2DM[7,8] and CVD,[9,10] and is founded on five metabolic risk markers: central obesity, elevated blood pressure (BP), dyslipidemia (involving both elevated serum triglycerides and lowered high-density lipoprotein (HDL) cholesterol), and elevated fasting glucose.[11,12] Insulin resistance, commonly believed to be originating from central obesity,[13] is considered the cornerstone in risk profiles describing both T2DM and CVD,[14] and central obesity has, therefore, with the 2005 International Diabetes Federation (IDF) definition, been set as the "entrance criteria" in defining MetS.[15,16] Many studies have described the association between MetS-based risk factors and subsequent disease risk; however, most studies are conducted on middle-aged populations.[10,17–19] There is, a need for studies on how the current MetS definition associates to disease risk specifically in elderly individuals. This study aimed to investigate the predictive value of MetS in relation to future risk of T2DM and CVD in a cohort of elderly Caucasian women by applying the

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MetS definition set by the IDF. This investigation would allow for an assessment of whether the MetS-based assessment criterion remains valid in the estimation of future increased risk of T2DM and CVD development also in an older population.

All present studies within the field of MetS report the risk estimate based on the use of a defined MetS-group compared to a reference group not defined with the syndrome. When applying this dichotomized definition, it is likely that the reference group will be heterogeneous and contain individuals who display variable metabolic profiles. Such reference group heterogeneity would be based on the inclusion of individuals who, while not meeting the central obesity entrance criterion, might still hold many other MetS risk factors, such as hypertension, dyslipidemia (elevated serum triglycerides and lowered HDL cholesterol), and hyperglycemia. We here hypothesized that a heterogeneous metabolic state of the reference group could potentially influence the syndrome's predictive power of disease. To test the influence of the reference group, we separated our study control group into three reference subgroups: centrally obese controls not defined with MetS, controls with no central obesity but other MetS risk factors, and controls with no MetS risk factors, and used principal component analysis (PCA) to visualize the differences between the MetS group and these reference subgroups. We further investigated whether the syndrome's predictive power of T2DM and CVD would increase when stratifying the reference group into the three subgroups of varying risk character. Finally, we also explored the disease risk profile of T2DM and CVD based solely on cumulating numbers of MetS risk factors.

2. Methods

2.1. Study population

The Prospective Epidemiological Risk Factor (PERF) study is an observational, prospective study of elderly Danish women (n = 5855) conducted in 1999 to 2001. The cohort consists of postmenopausal women who either had previously participated in clinical randomized placebo-controlled studies or were screened without being randomized for previous studies at the Center for Clinical and Basic Research (CCBR) in Copenhagen or Aalborg, Denmark. Prior studies run at CCBR, which ultimately lead to the study population in PERF, mainly focused on age-related diseases such as osteoporosis and osteoarthritis, and both screen failures and enrolled participants from these studies (n = 8875) were invited and included on equal terms in the PERF study. The study was carried out in accordance with ICH-GCP with study protocol approval from the local ethics committees; The Research Ethics Committee of Copenhagen County and the Research Ethics Committee of Viborg and North Jutland Counties, Denmark (approval reference: KA 99070gm). Written informed consent was obtained from all participants.

Baseline examination comprised a physical examination including a full-body dual-energy X-ray absorptiometry (DEXA) scan, blood sampling, and a self-reported questionnaire compiling information on smoking habits, alcohol intake, medical history, menopause age, physical activity level, and educational level.

2.2. Definition of the metabolic syndrome

MetS was defined using a modified version of the definition set by IDF.[15] Waist circumference was not directly measured in PERF, and therefore the definition of central obesity was based on a calculated central/peripheral fat mass ratio (C/P ratio) determined by DEXA scan. Central fat mass was defined as fat located at the torso and peripheral fat mass defined as fat located on arms, legs, and head as determined by DEXA scan. The cohort was divided into quartiles based on the C/P ratio, and only subjects in the fourth quartile were defined as centrally obese in the analysis. All subjects in this quartile had a C/P ratio >1.

The MetS inclusion criteria were defined as a C/P ratio >1 or a body mass index (BMI) >30 kg/m^2, and 2 or more of the following risk factors: increased triglycerides (>1.7 mmol/L), decreased HDL cholesterol (<1.29 mmol/L), increased fasting plasma glucose (>5.6 mmol/L), and increased BP (systolic >130 mm Hg or diastolic >85 mm Hg or treatment of previously diagnosed hypertension).

The IDF criteria state that treatment for lipid abnormalities specifically targeting HDL cholesterol or triglycerides can be used as defining the risk factor, rather than the actual serum value itself. However, as specified hyperlipidemia treatment was not part of the questionnaire, we were not able to determine the specific lipid-lowering treatments; therefore, only the serum measurements for these 2 variables were part of the MetS-defining criteria of dyslipidemia in this study.

2.3. Study endpoints

The study endpoints were a T2DM diagnosis or a CVD event occurring after participation in PERF. Follow-up information on T2DM and CVD diagnosis was retrieved from The National Danish Diabetes Registry and The National Danish Patient Registry, respectively, using a unique personal identification number for each subject. Classification of CVD diagnoses was completed according to The International Classification of Diseases, 10th revision (version 2016). All diagnoses from Chapter IX (Diseases of the circulatory system) were included in the analysis as CVD events.

The dataset used for analysis was defined as subjects with no missing data for all MetS-defining variables and no T2DM or CVD diagnosis before PERF (n = 3905) as illustrated in Fig. 1.

The maximum follow-up period was 15.1 years (mean follow-up: 12.7 ± 3.0 years) starting on the day of study enrollment and ending at either occurrence of an event (register-based diagnosis) or on December 31, 2014 (registry data retrieval date), whichever came first. Of the entire study population, a total of 762 diabetics were identified, whereof 229 subjects were excluded from the analysis due to diagnosis before study enrollment. CVD diagnosis was identified in 3744 subjects, whereof 1313 subjects were excluded for having a CVD event before study enrollment. Of these 1313 subjects, 69 were also diagnosed with diabetes before enrollment, leaving 1217 unique subjects excluded based solely on CVD event history.

One or several data points for defining MetS were missing for 446 subjects. Sixty-three subjects were underweight (BMI ≤ 18.5 kg/m^2), and thus, the DEXA scan may not be suitable for the definition of a relevant C/P ratio in this subgroup. In total, 509 subjects had either missing or inconclusive data points to permit definition of MetS. Altogether, 3905 subjects were included for further analysis.

In addition to the stratification based on identified MetS, data were analyzed based on a cumulative number of MetS risk factors (0–5) in order to investigate the cumulative effect of risk factors. In this regard, risk factors were dichotomized based on the cutoff for the MetS criteria.

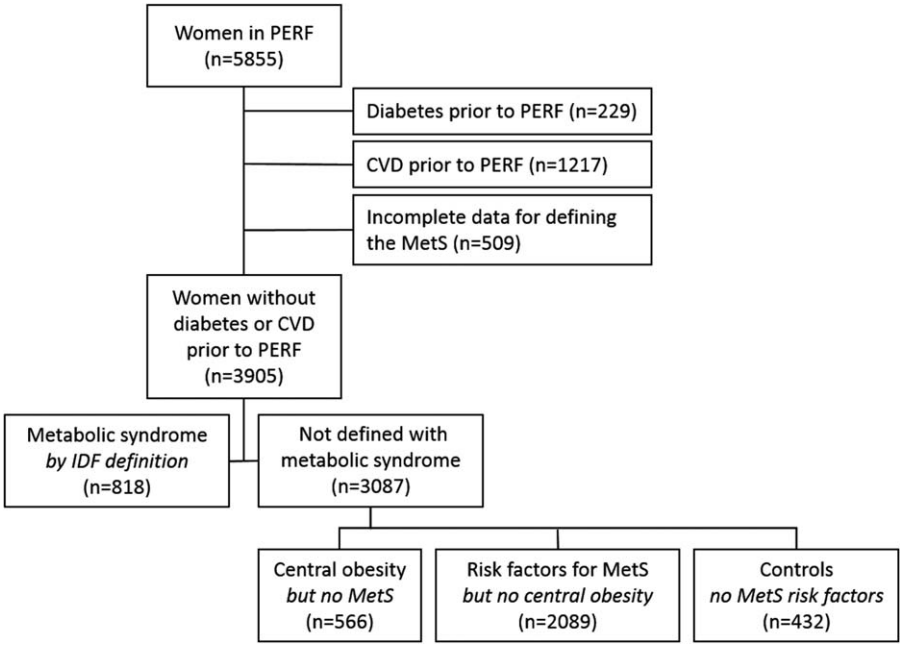

Figure 1. Definition of the study population. CVD = cardiovascular disease, MetS = metabolic syndrome, IDF = International Diabetes Federation, PERF = Prospective Epidemiological Risk Factor study.

2.4. Statistical analysis

Statistical analysis was conducted using MedCalc Statistical Software v. 14.8.1 (MedCalc Software, Ostend, Belgium), GraphPad Prism v.6 (GraphPad Software, La Jolla, CA), and SAS software, Version 9.4 (SAS Institute Inc., Cary, NC). PCA was performed in R v. 2.15.3 (R Development Core Team, Vienna, Austria) using the ggbiplot package.

Baseline characteristics of subjects with defined MetS compared to subjects with no risk factors for MetS (Table 1) were analyzed using Mann–Whitney *U* test (numerical variables) or chi-square test (categorical variables).

Multivariate Cox proportional hazards regression model with age as time scale was used to assess three aspects of the MetS: risk of developing T2DM and CVD in women defined with MetS compared to women not defined with the syndrome; risk associated with the individual MetS risk factors and subsequent T2DM or CVD (Fig. 2); risk of developing T2DM and CVD in women with defined MetS, in women with central obesity, and up to one additional MetS risk factor, but not defined with MetS, and in women with other risk factors for MetS than central obesity. Women holding no risk factors for MetS were used as the reference group (Fig. 4A). Categorical variables included in all multivariate Cox proportional hazard regression models were current smoking (yes/no), current alcohol consumption (<7 vs ≥7 drinks/wk), and physical activity other than walking (<2 vs ≥2 sessions/wk). The Cox proportional hazard regression model was further used to assess the risk of T2DM and CVD based on the cumulative number of metabolic risk factors (1–5), where subjects with no MetS risk factors were used as reference group (Fig. 4B). Incidence rates were calculated for all groups (Table 2) as incidence per 1000 person-years.

PCA (Fig. 3A) was computed from C/P ratio, BMI, triglycerides, HDL cholesterol, fasting glucose, systolic BP, diastolic BP, smoking status, alcohol consumption, physical activity, low-density lipoprotein (LDL) cholesterol, total cholesterol, white blood cell count (WBC), alanine-aminotransferase

Table 1

Cohort characteristics of elderly women with and without defined MetS.

	MetS by IDF definition (n = 818)	Controls not defined with MetS (n = 3087)
Demographics		
Age, y	70.5 (69.8–71.0)	70.3 (69.9–70.6)
Menopause age, y	50.0 (49.0–50.0)	50.0 (50.0–50.0)
Family history of diabetes (%)	8.5 (62)	8.1 (216)
Education		
Primary school (%)	71.0 (579)	69.9 (2158)
High school (%)	21.2 (173)	23.0 (709)
University (%)	7.8 (64)	7.1 (220)
Occupation (working, %)	76.0 (621)	74.7 (2303)
Lifestyle		
Current smoking (%)	20.8 (170)	22.6 (697)
Alcohol (>7 gl/wk, %)	31.2 (255)	34.5 (1059)
Physical activity (≥2 sessions/wk, %)	63.7 (521)*	73.5 (2269)
Vitals		
Height, cm	160.5 (160.2–161.0)	161.1 (160.8–161.3)
Weight, kg	76.4 (75.4–77.4)*	64.6 (64.2–65.1)
BMI, kg/m^2	30.0 (29.5–30.3)*	24.9 (24.8–25.1)
Systolic blood pressure, mm Hg	155.0 (153.0–156.0)*	145.0 (144.0–147.0)
Diastolic blood pressure, mm Hg	85.0 (84.0–86.0)*	80.0 (80.0–81.0)
Serum chemistry		
Glucose, mmol/L	5.8 (5.8–5.9)*	5.2 (5.2–5.2)
Cholesterol, mmol/L	6.4 (6.4–6.5)*	6.3 (6.3–6.3)
LDL, mmol/L	4.1 (4.0–4.2)†	3.9 (3.9–3.9)
HDL, mmol/L	1.5 (1.4–1.5)*	1.8 (1.8–1.8)
Triglycerides, mmol/L	1.8 (1.7–1.8)*	1.1 (1.1–1.2)
White blood cells, 10^9 cells/L	5.9 (5.8–6.1)*	5.4 (5.4–5.5)
ALAT, mmol/L	27.0 (26.0–27.0)*	23.0 (23.0–23.0)
ASAT, mmol/L	24.0 (24.0–25.0)*	23.0 (23.0–23.0)

Data shown as median value (95% confidence interval) or as percentage (absolute number of cases). ALAT = alanine-aminotransferase, ASAT = aspartate-aminotransferase, BMI = body mass index, HDL = high-density lipoprotein, IDF = International Diabetes Federation, LDL = low-density lipoprotein, MetS = metabolic syndrome.

* Significantly different from controls ($P < 0.001$).

† Significantly different from controls ($P = 0.007$).

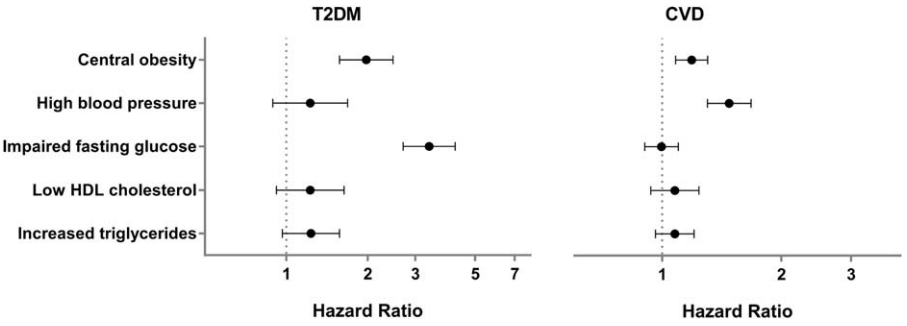

Figure 2. Risk associated with the 5 metabolic risk factors used to define the metabolic syndrome showed central obesity to be the only risk factor contributing to increased risk of both T2DM and CVD outcome. Multivariate Cox regression analysis for the risk of developing T2DM and CVD based on individual metabolic risk factors; central obesity, high blood pressure, elevated fasting glucose, decreased HDL cholesterol, and increased triglyceride levels. Values were adjusted for age, smoking, alcohol consumption, and physical activity. CVD = cardiovascular disease, T2DM = type 2 diabetes mellitus. Data represent hazard ratio with 95% confidence interval.

(ALAT), and aspartate-aminotransferase (ASAT). All variables were assessed for normality, and C/P ratio, BMI, and triglyceride levels were log-transformed to ensure normality in the data distribution. Subjects with a WBC serum levels $>10^9$ cells/L, or ALAT or ASAT levels >50 mmol/L, were excluded from the PCA (n = 161) to secure a representative presentation of the metabolic risk factor distribution in the cohort, so that subjects with extreme WBC, ALAT, and ASAT values would not distort the analysis. After centering and scaling the data, we obtained the principal components (PCs) describing the systematic variation in data across the 15 variables, hence revealing the metabolic profiles in the dataset. The differences between the PC1 components of the four groups were compared using one-way analysis of variance with 95% confidence limits. Tukey's test was applied as post hoc analysis to determine pairwise differences between groups (Fig. 3B). The relationship between subjects defined with MetS compared to the 3 non-MetS subgroups was also analyzed using a Kruskal–Wallis test (Supplemental Digital Content 1, http://links.lww.com/MD/B253). For P values less than 0.05, a post hoc test for pairwise comparison of subgroups, according to Conover,[20] was performed.

3. Results

3.1. Metabolic syndrome in elderly women

Among the elderly women in the PERF cohort, we found that 20.9% were defined having MetS (n = 818) (Table 1). The demographic characteristics, education level, and lifestyle did not vary among subjects with MetS and controls except for physical activity level, which was greater in the control group ($P < 0.001$).

Serum LDL and total cholesterol, which are lipid parameters not used in the MetS definition, varied significantly between the two groups ($P < 0.001$ and $P = 0.007$, respectively). This was also the case for WBC and the liver function markers ALAT and ASAT ($P < 0.001$ for all three variables).

We found a 3.6-fold increased risk of developing T2DM (hazard ratio (HR) = 3.63, 95% confidence interval: [2.93–4.48])

Table 2

Incidence rates of T2DM and CVD within elderly women in the PERF cohort stratified based on metabolic definitions or based on number of risk factors.

	Type 2 diabetes					CVD				
Groups	Person-years at risk	T2DM cases	Incidence per 1000 person-years	Lower 95% CI	Upper 95% CI	Person-years at risk	CVD cases	Incidence per 1000 person-years	Lower 95% CI	Upper 95% CI
MetS by IDF definition	8993.4	164	18.2	15.7	21.3	7292.5	487	66.8	61.1	73.0
Central obesity but no MetS	6923.7	44	6.4	4.7	8.5	5361.6	316	58.9	52.8	65.8
Risk factors for MetS but no central obesity	25,504.5	127	5.0	4.2	5.9	19,875.5	1093	55.0	51.8	58.4
Controls not defined with MetS	5636.6	16	2.8	1.7	4.6	4632.2	168	36.3	31.2	42.2

	Type 2 diabetes					CVD				
Number of risk factors	Person-years at risk	T2DM cases	Incidence per 1000 person-years	Lower 95% CI	Upper 95% CI	Person-years at risk	CVD cases	Incidence per 1000 person-years	Lower 95% CI	Upper 95% CI
5	662.3	24	36.2	24.3	54.1	520.4	50	96.1	72.8	126.8
4	2963.8	62	20.9	16.3	26.8	2387.5	163	68.3	58.6	79.6
3	7332.5	99	13.5	11.1	16.4	6011.3	364	60.6	54.6	67.1
2	13,001.0	90	6.9	5.6	8.5	10,040.7	601	59.9	55.3	64.8
1	17,461.9	60	3.4	2.7	4.4	13,569.6	718	52.9	49.2	56.9
0	5636.6	16	2.8	1.7	4.6	4632.2	168	36.3	31.2	42.2

CI = confidence interval, CVD = cardiovascular disease, IDF = International Diabetes Federation, MetS = metabolic syndrome, T2DM = type 2 diabetes mellitus.

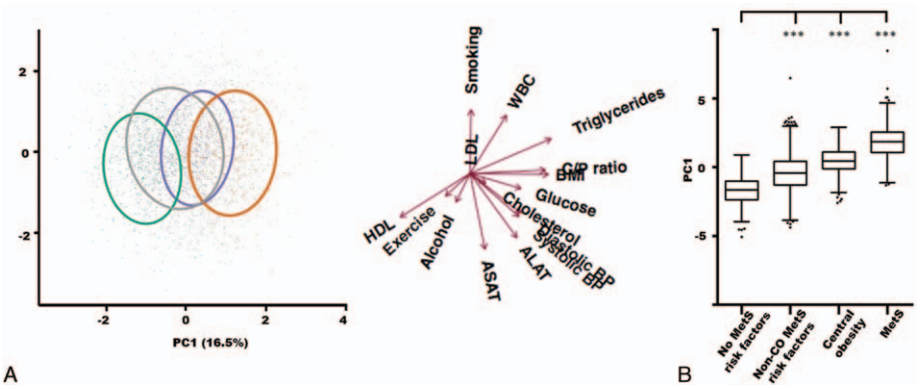

Figure 3. A heterogeneous metabolic risk profile within the control group. (A) Principal component analysis score plot colored by group: reference group subjects with no metabolic risk factors (green), subjects with risk factors for MetS but no central obesity (gray), subjects with central obesity and up to 1 other MetS risk factor (purple), and subjects with defined MetS (orange). The ellipses cover 68% of the subjects belonging to a given subgroup. Loadings for the included parameters are shown with arrows. ALAT = alanine-aminotransferase, ASAT = aspartate-aminotransferase, C/P ratio = central/peripheral fat mass ratio, cholesterol = total cholesterol, glucose = fasting glucose, WBC = white blood cell count. Exercise: physical activity. (B) Distribution of the principal component 1 scores for the 4 subgroups. Boxes represent the upper quartile, the mean, and the lower quartile of the data. Whiskers designate the Tukey interval with outliers shown as staggered dots. $^{***}P < 0.001$. MetS = metabolic syndrome, non-CO = non-central obese.

and a 1.3-fold increased risk of a CVD event (HR = 1.29 [1.16–1.43]) after 12.7 ± 3.0 years of follow-up for subjects with MetS compared to controls without defined MetS. Given the strong effects of MetS on disease risk, we further investigated the relationship between the individual MetS risk factors and subsequent T2DM or CVD events (Fig. 2).

Central obesity was the only MetS risk factor contributing to increased risk of both outcomes with a 2-fold increased risk of T2DM (HR = 1.98 [1.57–2.48]) and a 1.5-fold increased risk of a CVD event (HR = 1.48 [1.30–1.68]) (Fig. 2). Elevated fasting glucose was only related to the development of T2DM (HR = 3.38 [2.71–4.22]) and did not contribute to an increased risk of

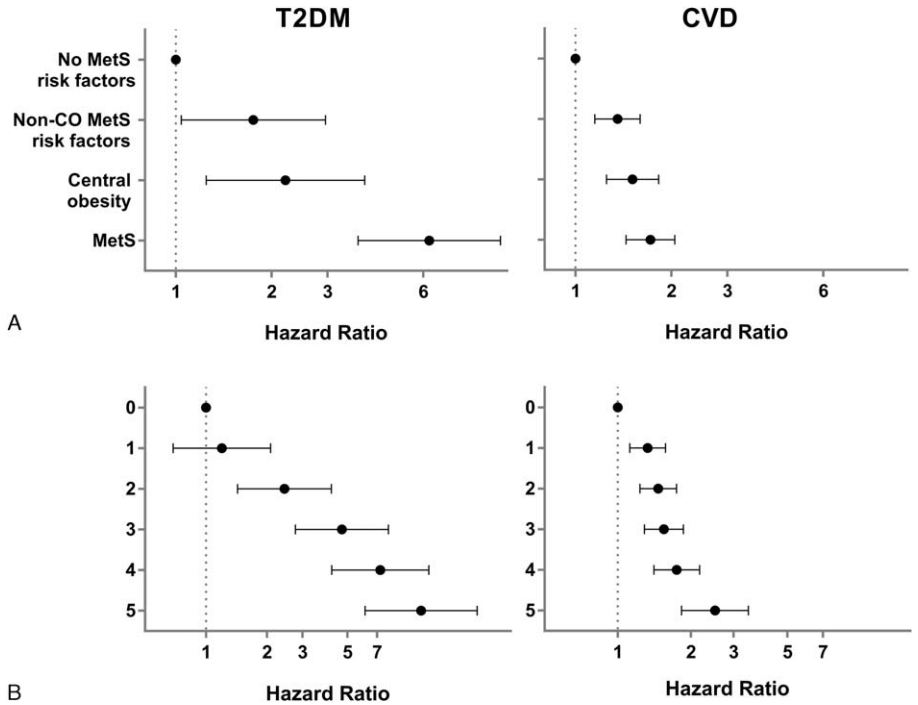

Figure 4. Stratification of the heterogeneous control group in identified intermediate subgroups with increased risk for later T2DM and CVD. (A) Multivariate Cox regression analysis for the risk of developing T2DM and CVD based on control group stratification. Subgroups represent reference group with no risk factors for MetS, subjects with risk factors for MetS but no central obesity, subjects with central obesity and up to one additional MetS risk factor, and subjects with defined MetS. Values are adjusted for age, smoking, alcohol consumption, and physical activity. CVD = cardiovascular disease, MetS = metabolic syndrome, non-CO = non-central obesity, T2DM = type 2 diabetes mellitus. (B) Risk of developing T2DM and CVD for subjects with 1 to 5 risk factors for MetS as compared to the reference group with no MetS risk factors. 0: n = 432; 1: n = 1404; 2: n = 1083; 3: n = 647; 4: n = 271; and 5: n = 68. Values are adjusted for age, smoking, alcohol, and physical activity. Data represent hazard ratio with 95% confidence interval.

CVD. Conversely, high blood pressure was a contributor to the development of CVD events (HR = 1.19 [1.09–1.30]) but did not contribute to an increased risk of T2DM. Neither HDL cholesterol nor triglyceride levels contributed to an increased risk of T2DM and CVD in this cohort of elderly Caucasian women.

3.2. Subgrouping the control group consisting of subjects with heterogeneous MetS risk factor profiles

Since central obesity alone contributed to increased risk of both T2DM and CVD, we speculated if the subjects with central obesity in the control group would take part in reducing the prediction of future disease prevalence within defined MetS subjects. To examine this question, we divided the heterogeneous control group into 3 subgroups: subjects with central obesity, and up to 1 additional MetS risk factor, but not defined with MetS; subjects without central obesity, but with other risk factors for the MetS; and subjects with no MetS risk factors.

To capture the multivariate features of the dataset, we used PCA to visualize the differences between the MetS group and the three control subgroups (Fig. 3A). We observed a distinct separation between subjects with defined MetS (orange) and the control group comprising subjects with no risk factors for MetS (green), while the non-MetS subjects with central obesity (purple) and subjects with other MetS risk factors (gray) cut in between the non-MetS risk factor controls and MetS subjects in the PCA score plot. Based on the group distributions, the multivariate analysis indicated that subjects with central obesity and up to 1 MetS risk factor are metabolically more similar to MetS subjects, while subjects with other MetS risk factors than central obesity are more similar to the reference group with no MetS risk factors. The 4 subgroups were found to statistically separate in PC1 (Fig. 3B), meaning that all subgroups differed in the parameters pulling in the PC1 direction within the loading plot. The parameters driving this separation are mainly MetS classification parameters such as C/P ratio, BMI, fasting glucose, HDL cholesterol, triglycerides, and blood pressure. Smoking, LDL cholesterol, and ASAT had no influence on the separation of the subjects in PC1.

Since the PCA indicated that the three subgroups from the former control group showed differentiated metabolic profiles, we used Cox regression analysis to investigate whether these subjects also showed different risk profiles for T2DM and CVD. We found that controls with central obesity without MetS had a 2.2-fold increased risk of T2DM (HR = 2.21 [1.25–3.93]) and a 1.5-fold increased risk of CVD (HR = 1.51 [1.25–1.83]) compared to the reference group with no risk factors for MetS (Fig. 4A). Likewise, controls with other MetS risk factors than central obesity had a 1.8-fold increased risk of T2DM (HR = 1.75 [1.04–2.96]) and a 1.4-fold increased risk of CVD (HR = 1.36 [1.15–1.60]). Moreover, the stratification of the former control group also affected the disease risk in MetS subjects, as subjects with defined MetS showed a 6.3-fold increased risk of developing T2DM (HR = 6.29 [3.74–10.50]) and a 1.7-fold increased risk of a CVD event (HR = 1.72 [1.44–2.05]), when specifically compared to the reference group without MetS risk factors.

Further, we explored the effect of the risk factor distribution further by analyzing the relationship between the cumulated sum of risk factors and subsequent disease events. The average number of MetS risk factors for all subjects in the analytical sample was 1.8 ± 1.2. T2DM risk was increased for subjects with ≥2 MetS risk factors compared to subjects with no risk factors; 1 risk factor: HR = 1.20 (0.69–2.09), 2 risk factors: HR = 2.44 (1.43–4.17), 3 risk factors: HR = 4.70 (2.77–7.98), 4 risk factors: HR = 7.27 (4.19–12.61), and 5 risk factors: HR = 11.57 (6.12–21.88), respectively (Fig. 4B). An increased risk of a CVD event was found with ≥1 risk factor for MetS: HR = 1.33 (1.12–1.58), HR = 1.47 (1.24–1.75), HR = 1.55 (1.29–1.86), HR = 1.75 (1.41–2.18), and HR = 2.52 (1.83–3.46), respectively, as illustrated in Fig. 4B. The incidence rates shown in Table 2 further manifested the differentiated risk within the metabolic subgroups when stratified either based on metabolic definitions or based on number of risk factors. The lowest incidence was found in the control group holding no risk factors for MetS, with an incidence of 2.8 (1.7–4.6) per 1000 person-years for T2DM and an incidence of 36.3 (31.2–42.2) per 1000 person-years for CVD. The highest incidence was found in the group holding 5 risk factors for MetS, with an incidence of 36.2 (24.3–54.1) per 1000 person-years for T2DM and an incidence of 96.1 (72.8–126.8) per 1000 person-years for CVD.

4. Discussion

Elderly women with MetS proved to have an increased risk of developing T2DM and CVD when compared to women not defined with the syndrome. The increased risk of 3.6-fold for T2DM and 1.3-fold for CVD found in this study correlated well with findings reported in previous studies using a heterogeneous control group, although these results mostly originate from cohorts of middle-aged men and women.[10,17–19] We further refined these results by highlighting how a control group with heterogeneous MetS risk profiles in women without defined MetS can lead to a distortion of the hazard estimations associated with the MetS. We showed how specifically comparing subjects with defined MetS to subjects with no risk factors for MetS increased the risk estimate of future T2DM from 3.6 to 6.3-fold and the risk of a future CVD event from 1.3 to 1.7-fold. This clearly suggests that the risk of developing T2DM and CVD in women with defined MetS is much greater than previously proposed and further, that the risk of T2DM and CVD also was greater in women not defined with the syndrome but still holding some risk factors for MetS. To our knowledge, this type of risk assessment of the MetS has not previously been reported. In addition, the analysis of cumulating MetS risk factors showed increasing risk of later disease with increasing number of risk factors; with 5 MetS risk factors resulting in 11.6-fold increased the risk of T2DM development and 2.5-fold risk of CVD. This underlines the value of identifying subjects with MetS risk factors in the elderly population as well.

Central obesity was the only MetS risk factor that independently contributed to the risk of both future T2DM and CVD (2- and 1.5-fold, respectively). As central obesity is consistently highlighted as a key contributor to risk in any definition of the MetS,[16] our finding is congruent with this prominent role of central obesity in the MetS definition. By partitioning the control group of non-MetS subjects into 3 subgroups, we repeated our finding of a 2-fold increased risk of T2DM and 1.5-fold for CVD outcomes in subjects with central obesity without MetS. Furthermore, the PCA revealed that subjects with central obesity displayed a higher degree of similarity to MetS subjects than the 2 other subgroups without this risk factor, emphasizing the role of central obesity as a key driver of both T2DM and CVD. While we clearly demonstrated the predictive value of the MetS in relation to later risk of T2DM and CVD in elderly Caucasian women,

we also showed that women not fulfilling the full MetS criteria likewise have a higher risk of developing T2DM and CVD later in life, if they have one or more of the MetS risk factors at baseline. This was further illustrated in the differentiated incidence rates found within the subdivided reference group. Further, the calculated incidence rates also underlined how the incidence of both T2DM and CVD increased with increasing numbers of risk factors.

The prognostic importance of the MetS compared to the prognostic capability of the sum of the individual MetS risk factors has previously been challenged by others.[21–23] With the PCA and risk estimates presented in this study, we add to this debate by assessing the risk of the individual components, highlighting the heterogeneity in the metabolic profiles of subjects not defined with MetS, and determining the predictive ability of the cumulating sum of risk factors constituting the MetS. Other studies have compared the predictive ability for CVD using both the MetS definition and the Framingham Risk Score[24–26] finding similar results for the two scoring systems, and further found the Diabetes Prediction Model to be superior to the MetS definition in predicting the risk of diabetes development.[25] Similarly, the findings in our study indicated that defining the MetS does not supersede the risk estimated when summing the risk of the individual risk factors. Consequently, our findings add to the questioning of applying a MetS definition to commonly cooccurring risk factors will provide auxiliary value in the general practice. Thus, it might be more practical to focus on developing a classification scheme that reflects both the degree and sum of risk factor abnormalities instead of using the current MetS definition. This suggestion is founded on the assumption that cooccurring factors indeed enhance the risk of adverse outcomes, as was also the result of our current cumulating risk factor analysis.

Regardless of focusing on MetS as a joined definition or on the sum of risk factors, it is known that the prevalence of the risk factors for MetS increases with age, reaching a prevalence of 40% in people aged >60 years.[2] The initial indicator of a high-risk metabolic profile is central obesity, and our present study coherently points to the high priority of this risk factor in the elderly segment of the population, when focusing on preventing T2DM and CVD and in advancing efforts to regulate the obesity epidemic.

The strengths of this study include its longitudinal design, detailed assessment of metabolic risk factors, and exclusion of subjects with T2DM and CVD at baseline. The study's follow-up information was derived from Danish registry data, which is of high quality based on the use of a unique personal identifier and nationwide electronic patient records, and thus results in limited loss of data from baseline to follow-up. The cohort consists of a large group of women in Denmark, where the homogenous population with equal access to primary care (tax-paid, not individually paid) may limit extrapolations to other populations. However, the hazard ratios found in this study are comparable to associations found in similar cohorts, though with different age distributions, which indicates that such generalizations are indeed plausible. By applying PCA as a multivariate tool to assess risk profiles, we introduce a possible confounder, as we subdivide the study population before PCA based on central obesity. With this common denominator being present in both the MetS group and the non-MetS group with central obesity, we potentially skew these 2 subgroups toward each other compared to the non-MetS group holding other risk factors for MetS, as this subgroup may be regarded as being more heterogeneous (by not having obesity as a common denominator). However, based on the MetS definition, it is not possible to circumvent this type of limitation. In this study, central obesity was determined by DEXA scan rather than waist circumference originally proposed by IDF. However, IDF does highlight that DEXA scan can be used as an additional factor in research of the MetS, which can allow further modification of the definition if necessary.

Elderly Caucasian women fulfilling the MetS criteria set by the IDF showed increased risk of future T2DM or CVD diagnosis; however, subjects who did not fulfill the criteria for MetS but presented one or more of the components of MetS were also at increased risk. A further subdivision of the reference group proved to increase the risk of T2DM to 6.3-fold (from 3.6-fold) and 1.7-fold for CVD (from 1.3-fold) for MetS subjects when compared to a reference group only including subjects with no MetS risk factors. In clinical practice, employment of the MetS in elderly women should be focused as a tool for identifying subjects with metabolic high-risk profiles. However, the sum of risk factors are proposed to be equally considered, as subjects not fitting the MetS-criterion, but still holding one or more risk factors for MetS, were here identified also to be at increased risk of T2DM and CVD.

Acknowledgements

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CC serves as a board member and stock owner in Nordic Bioscience. MAK and KH hold stocks in Nordic Bioscience.

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Supplemental Digital Content 1: Cohort characteristics of PERF participants with MetS and subgroups not defined with metabolic syndrome
Data shown as median value (95% confidence interval) or as percentage (absolute number of cases).

	N	**Metabolic Syndrome** *by IDF definition* n = 818	N	**Central obesity** *but no MetS* n = 566	N	**Risk factors for MetS** *but no central obesity* n = 2089	N	**Controls** *not defined with MetS* n = 432	**P-value**
Demographics									
Age (years)	818	70.4¤(69.8-71.0)	566	70.0¤ (69.3-70.6)	2089	71.0§¤ (70.6-71.4)	432	66.6§ (66.0-67.3)	<.001
Menopause age (years)	804	50.0 (49.0-50.0)	560	50.0 (50.0-50.0)	2069	50.0 (50.0-50.0)	428	50.0 (49.2-50.0)	.16
Family history of diabetes (%)	731	8.5 (62)	507	8.2 (41)	1795	7.9 (142)	376	8.8 (33)	.93
Education									
Primary school (%)	816	71.0 (579)	566	74.0¤ (419)	2089	69.6 (1454)	432	66.0 (285)	.04
High School (%)	816	21.2 (173)	566	19.8 (112)	2089	23.1 (482)	432	26.6 (115)	.052
University (%)	816	7.8 (64)	566	6.2 (35)	2089	7.3 (153)	432	7.4 (32)	.70
Occupation (Working, %)	817	76.0 (621)	566	75.4 (427)	2087	74.1 (1547)	430	76.5 (329)	.60
Vitals									
Height (centimeter)	818	160.5¤(160.2-161.0)	566	160.4¤ (159.9-160.8)	2089	161.2¤ (160.9-161.4)	432	161.8§ (161.2-162.4)	.0012
Weight (kg))	818	76.4¤ (75.4-77.4)	566	72.3§¤ (71.2-73.5)	2089	63.6§¤ (63.2-64.1)	432	61.9§(60.9-62.7)	<0.001$
BMI (kg/m²)	818	30.0¤ (29.5-30.3)	566	28.1§¤ (27.6-29.1)	2089	24.6§¤ (24.4-24.8)	432	23.6§ (23.3-23.9)	<.001$
Systolic blood pressure	817	155.0¤ (153. 0-156.0)	565	150.0§¤ (146.0-152.0)	2087	150.0§¤ (149.0-151.0)	432	120.0§ (119.0-120.0)	<.001$
Diastolic blood pressure	817	85.0¤ (84.0-86.0)	565	83.0§¤ (81.0-84.0)	2087	83.0§¤ (82.0-83.8)	432	72.0§ (70.0-73.0)	<.001
Lifestyle									
Current smoking (%)	818	20.8¤ (170)	566	18.4¤ (104)	2089	22.3¤ (466)	432	29.4§ (127)	<.001
Alcohol (>7gl/week, %)	817	31.2 (255)	560	32.5 (182)	2079	34.5 (717)	428	37.4 (160)	0.12
Physical activity (≥2 sessions/week, %)	818	63.7¤ (521)	566	69.3¤ (392)	2088	73.8§ (1541)	432	77.8§ (336)	<.001
Serum chemistry and hematology									
White blood cells (10^9cells/L)	818	5.9¤ (5.8-6.1)	565	5.4§ (5.3-5.6)	2085	5.4§¤ (5.4-5.5)	432	5.3§ (5.2-5.4)	<.001
Glucose (mmol/L)	818	5.8¤ (5.8-5.9)	566	5.2§¤ (5.2-5.3)	2089	5.3§¤ (5.2-5.3)	432	5.0§¤ (4.9-5.1)	<.001$
Cholesterol (mmol/L)	818	6.4¤ (6.4-6.5)	566	6.2§ (6.1-6.3)	2089	6.4¤ (6.3-6.4)	432	6.2§ (6.1-6.3)	<.001
LDL (mmol/L)	818	4.1¤ (4.0-42)	566	3.9§¤ (3.8-4.0)	2088	3.9§¤ (3.9-4.0)	432	3.7§ (3.6-3.8)	<.001
HDL (mmol/L)	818	1.5¤ (1.4-1.5)	566	1.7§¤ (1.7-1.8)	2089	1.8§¤ (1.8-1.8)	432	1.9§ (1.8-1.9)	<.001$
TRIG (mmol/L)	818	1.8¤ (1.7-1.8)	566	1.2§¤ (1.2-1.2)	2089	1.2§¤ (1.1-1.2)	432	1.0§ (1.0-1.1)	<.001
ALAT (mmol/L)	818	27.0¤ (26.0-27.0)	566	24.0§¤ (24.0-25.0)	2089	23.0§¤ (22.0-23.0)	432	21.0§ (21.0-22.0)	<.001$
ASAT (mmol/L)	818	24.0¤ (24.0-25.0)	566	23.0§¤ (23.0-24.0)	2089	23.0§¤ (23.0-24.0)	432	23.0§ (22.0-23.0)	<.001

§ significantly different (P<.05) from subjects with defined MetS
$ all groups significantly different from each other
¤ significantly different (P<.05) from subjects with no risk factors for MetS

Additional Results (not published)

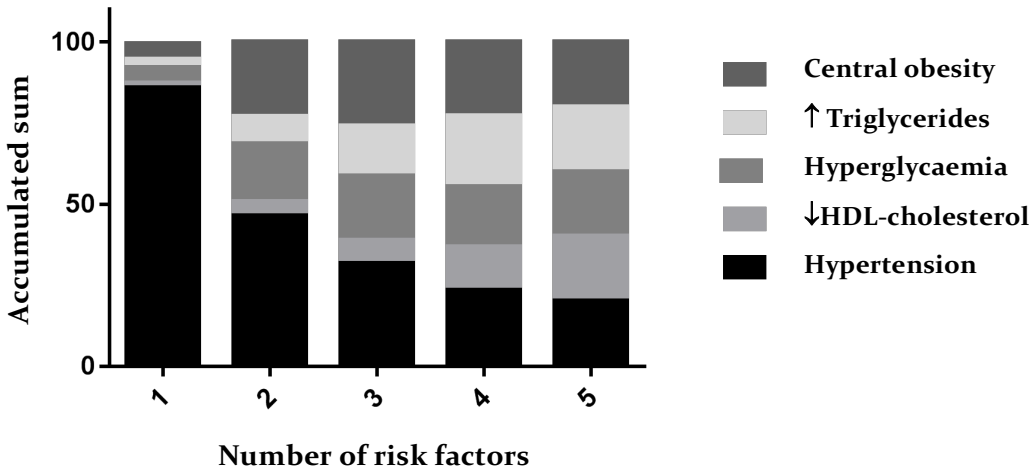

Figure – additional: Accumulated sum (%) of the five risk factors defining the MetS for subjects with 1, 2, 3, 4 or 5 risk factors for MetS. 0: n=432, 1: n=1404, 2: n=1083; 3: n=647, 4: n=271; 5: n=68. Entrance criteria: 1: 4.8%, 2: 23.0%, 3: 25.9%, 4: 22.8%, 5: 20.0%. Triglycerides: 1: 2.6%, 2: 8.5%, 3: 15.3%, 4: 21.9%, 5: 20.0%). Fasting glucose: 1: 4.8%, 2: 17.8%, 3: 20.0%, 4: 18.6%, 5: 20.0%. HDL cholesterol: 1: 1.4%, 2:4.3%, 3: 7,1%, 4: 13.3%, 5: 20.0%. Blood pressure: 1: 85.8%, 2: 46.4%, 3: 31,6%, 4: 23.4%, 5: 20.0%).

6 WEIGHT CHANGE AND HYPERGLYCAEMIC RISK

Title

Weight Change and Risk of Hyperglycaemia in Elderly Women

Aim

The aim of the study was to investigate the interplay between weight change and risk of hyperglycaemia in elderly women.

Rationale

Hyperglycaemia increases the risk of T2DM, heart disease, and stroke and is influenced by weight. It has been proposed that the association between BMI and the development of hyperglycaemia and T2DM is more complex than just a dose-response relationship and the impact of preceding weight change on blood glycemia levels in late-life is not well understood.

Findings

Overweight and obese elderly women with stable weight at follow-up presented an increased risk of hyperglycaemia, OR=2.2 [95% CI: 1.3-3.8] and OR=2.0 [1.0-4.2], compared to weight-stable women with normal weight at baseline. Overweight and obese women who lost weight during the follow-up period decreased their risk of hyperglycaemia to a level comparable with weight-stable normalweight women, whereas weight gain resulted in a 2.7-fold increased risk in overweight gainers and a 3.2-fold increased risk in obese weight gainers.

Conclusions

Losing weight in late life had a positive effect on the risk of hyperglycaemia in overweight and obese women, while further weight gain increased the risk of hyperglycaemia. The study highlights that strategies to reduce weight in obese and overweight elderly women could have a positive influence on hyperglycaemia and following disease burden in late-life.

Original Article

Weight Change and Risk of Hyperglycaemia in Elderly Women

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Keywords: Hyperglycaemia, weight, weight change, women, aging, epidemiology

ABSTRACT (250 words)

Background Hyperglycaemia increases the risk of type 2 diabetes, heart disease, and stroke and is influenced by weight. However, the impact of preceding weight change on blood glycemia levels in late-life is less well understood.

Aim We studied the interplay between weight change and risk of hyperglycaemia in a prospective cohort of elderly women.

Methods Elderly Caucasian women (age: 67.1 years at baseline, n=1,173) enrolled in the Prospective Epidemiological Risk Factor (PERF) study with baseline and 13-year follow-up measurements of BMI and fasting glucose levels (FPG) and no previous history of diabetes or impaired fasting glucose. Multivariate logistic regression was used to determine risk of hyperglycaemia (FPG≥5.6mmol/L or HbA1c≥42mmol/mol) in normalweight (BMI≤25kg/m2), overweight (BMI=25-29.9kg/m2) and obese (BMI≥30kg/m2) women who either lost weight, were weight-stable or had gained weight at follow-up.

Results Overweight and obese elderly women who had gained weight at follow-up presented an increased risk of hyperglycaemia, OR=2.7 [1.6-4.6] and OR=3.2 [1.5-6.8], compared to weight-stable normalweight women. Overweight and obese women who lost weight decreased their risk of hyperglycaemia to a level comparable to weight-stable normalweight women. Overweight and obese women with stable weight presented a 2-fold increased risk of hyperglycaemia compared to normalweight weight-stable women.

Conclusions Losing weight in late life had a positive effect on the risk of hyperglycaemia in overweight and obese women, while further, weight gain increased the risk of hyperglycaemia. The study highlights that strategies to reduce weight in obese and overweight elderly women could have a positive influence on disease burden in late-life.

MAIN TEXT

Hyperglycaemia is a risk factor for type 2 diabetes, heart disease, and stroke at all ages (1,2). With the increasing number of elderly people, of which many are chronically ill, the change in aging demographics is now considered a global burden (3). Obesity-induced hyperglycaemia in late life is a risk factor that greatly contributes to chronic illnesses, which justifies the eligibility of a cohort study of weight change and hyperglycaemia in the old age group (80+ years).

Hyperglycaemia is the first clinical symptom of type 2 diabetes resulting from defects in insulin production, insulin action, or both (4). Impaired glucose tolerance and impaired fasting glucose levels are important risk factors for the development of future diabetes and compared to subjects with normal glucose tolerance, the risk of type 2 diabetes increase 10-20 fold when hyperglycaemia is present (5). This increased risk does not seem to vary with age (6), highlighting the importance of continuous glucose monitoring even in late life.

The major driver of prediabetes and diabetes, besides age, is obesity and the obesity prevalence is concurrently increasing until 60 years of age. The Scottish Health Survey has in 2014 presented that while the overall prevalence of obesity showed little increase in the population aged 16-64, the body mass index (BMI) continued to rise specifically in women between age 55 and 74 with 78% being overweight or obese. This was 72% for the oldest age group (+75) (7). While diabetes risk studies are mainly focused on mid-life populations, elderly with diabetes have increased disease burden due to greater risk of acute and chronic microvascular and cardiovascular complications (8) which are linked to increased mortality, reduced functionality, and increased risk of institutionalization (9). Centers for Disease Control and Prevention propose that even if diabetes incidence will stagnate in the coming years, the prevalence of diabetes will double in the next 20 years, in part due to the aging of the population (10). The elder age group (>65 years of age) already presents the highest prevalence of diabetes globally (10), and therefore, effective strategies to reduce diabetes development, also in elderly, are highly warranted.

Here we aimed to investigate the influence of baseline weight and weight change during a period of 13-years on the subsequent risk of hyperglycaemia development in elder women. Subjects were 67(standard deviation 0.3) years of age at baseline and took part in the Prospective Epidemiological Risk Factor Study, which is a community-based cohort in Denmark.

RESEARCH DESIGN AND METHODS

The Prospective Epidemiological Risk Factor (PERF) study was an observational, prospective study of elderly Danish women conducted in 1999-2001 (PERF I) (n=5,855) with a follow-up visit (PERF II) in 2014-2015 (n=2,103). A full description of the cohort has been published elsewhere (11). The cohort consists of postmenopausal women who had either previously participated in clinical randomized placebo-controlled studies or were screened without being randomized for previous studies at the Center for Clinical and Basic Research (CCBR) in Copenhagen or Aalborg, Denmark. Prior studies run at CCBR, which ultimately lead to the study population in PERF, mainly focused on age-related diseases. Both screen failures and enrolled participants from these studies (n=8,875) were invited and included on equal terms in the PERF study. The participation rate was 72% in PERF I and 75% in PERF II. The baseline and follow-up visits comprised a physical examination, including height and weight measurement, and blood sampling performed by trained staff. Further, a self-reported questionnaire compiling information on medication, smoking status, alcohol intake, educational level, and physical activity. Both baseline and follow-up visits were carried out in accordance with Good Clinical Practice following the Helsinki declaration (ICH-GCP) with study protocol approval from The Research Ethics Committee of Copenhagen County (approval reference for PERF I: KA 99070gm and for PERF II: H-2-2012-074).

Study population

Elderly Caucasian women with baseline and follow-up data on BMI and fasting plasma glucose levels were included in this study. The dataset used for analysis (n=1,173) excluded women with missing BMI data (n=169), a pre-existing diabetes diagnosis prior to the follow-up visit (n=222), self-reported use of

antidiabetic medication in women who did not have a diagnosis elsewhere (n=5), fasting plasma glucose levels ≥5.6mmol/L at baseline (n=528) or missing data on plasma glucose levels (n=6). Information on pre-existing diabetes diagnosis was retrieved from The Danish National Diabetes Register (NDR) using a unique personal identification number for each subject. NDR is based on information from Danish health registries, including the Danish National Patient Registry, the Danish National Prescription Registry, and the Danish National Health Service Registry. The study endpoint was pre-diabetic hyperglycaemia determined as a fasting plasma glucose level ≥5.6mmol/L (≥100 mg/dL), or a pre-diabetic HbA1c-level ≥5.7% (≥42mmol/L) as defined by ADA (12), measured at the follow-up visit. At follow-up 447 women had hyperglycaemia of which 29 women had diabetic glucose levels with a fasting plasma glucose level >7.0mmol/L (>126 mg/dL) or an HbA1c-level >6.5% (>48mmol/L) (**Figure 1**). Fasting plasma glucose was measured directly after collection in both PERF I and II, using a Vitros 250 slide cartridge with no reagent system from Ortho Clinical, in PERF I, and an enzymatic measurement method using the Avida 1800, from Siemens, in PERF II.

BMI characteristics

Height and weight were recorded at baseline and follow-up with BMI calculated at both visits (weight (kg)/height squared (m^2)). Height was measured using a Charder transportable stadiometer and weight was measured using a Seca 899 class III standardized scale with yearly calibration. Trained staff performed the measurements. The BMI classification at baseline was done according to WHO definition (13) with BMI criteria for underweight: BMI≤18.5 kg/m^2 (n=19), normal weight: BMI>18.5-24.9 kg/m^2 (n=589), overweight: BMI=25.0-29.9 kg/m^2 (n=427) and obesity: BMI≥30 kg/m^2 (n=138). The underweight and normal weight women were combined in one group for logistic regression analysis and denoted normalweight. Each baseline BMI group was stratified in tertiles based on delta-BMI from baseline to follow-up to reflect BMI change. Women in the lower tertile group were denoted weight-losers, women in the middle tertile weight-stable and women in the upper tertile weight-gainers.

Spearman's correlation test was used to correlate BMI at follow-up with waist circumference, hip circumference, waist-hip ratio and muscle strength at follow-up. Tape measuring was used for

determining waist and hip circumference. The measuring band was applied midway between the inferior margin of the last rib and the crest of the ileum, in a horizontal plane. The hip was measured at the widest point in a horizontal plane. Muscle strength was measured by Jamar handgrip dynamometer using an average of three continuous measurements on the dominant hand. Trained health professionals carried out the measurements.

Statistical analysis

Statistical analysis was conducted using MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium).

Women who developed hyperglycaemia were compared to women who did not develop hyperglycaemia at baseline and follow-up using an independent two-way t-test for numerical variables following Welch test in case of unequal variance and a Chi-square test for categorical variables at baseline and follow-up.

Further, logistic regression analysis was applied to assess the association between BMI change and risk of a hyperglyacemic outcome. Subjects defined as normalweight at baseline with a stable weight at follow-up was used as reference group. Covariates known from the literature to influence the risk of hyperglycaemia was included in the multivariate regressions. In the partly adjusted model, adjustments were made for age at baseline and the categorical variables; educational level (primary school (ref), high school, university), smoking (never (ref), past, present), current alcohol consumption (<10.5 units/week vs. ≥10.5 units/week, with 10.5 units equal to 7 drinks) and, physical activity other than walking (<2 times/week vs. ≥2 times/week) at baseline. In the fully adjusted model, additional variables were added; systolic and diastolic blood pressure (per 10 mmHg increase), fasting glucose level at baseline, LDL- and HDL-cholesterol, triglycerides, self-reported treatment of hypertension (yes/no) and dyslipidaemia (yes/no), and self-reported family history of diabetes (yes/no). The Hosmer-Lemeshow test was applied to test for goodness of fit for the logistic regression model. The test indicated a good model fit based on the resulting low Chi-squared value.

RESULTS

Physiological changes from baseline to 13-years follow-up

The elderly women in the PERF study who developed hyperglycaemia in late life already showed significantly increased fasting glucose levels at baseline compared to women staying normoglycaemia at follow-up, yet still within the normoglycemic range (5.1 mmol/L vs. 5.0 mmol/L, P<0.0001). A larger fraction of the normoglycemic women was normalweight at baseline compared to women with future hyperglycaemia (5.4% vs. 41.8%, P<0.0001). At follow-up, the overall BMI were found to be slightly decreasing for normoglycemic women (from 25.1 kg/m^2 to 24.9 kg/m^2 from baseline to follow-up, P=0.07) whereas hyperglycemic women showed an overall increase in BMI during the follow-up period (from 25.9 kg/m^2 to 26.6 kg/m^2, P<0.001). In addition, 61.3% of the hyperglycemic women were either overweight or obese compared to 43.0% in the normoglycemic group at follow-up (P<0.001 for both BMI groups) (**Table 1**).

Other markers used to define metabolic dysfunction such as systolic blood pressure, HDL-cholesterol and triglycerides were significantly different in future hyperglycemic women as compared to normoglycemic women already at baseline (P<0.001 for all). HDL-cholesterol was still significantly lower and triglycerides significantly higher in hyperglycemic women at follow-up (P>0.0001 for both) (**Table 1**).

Alcohol consumption and physical activity level were not significantly different between the two groups at either baseline or follow-up. However, the fraction of current smokers was significantly higher in the hyperglycaemic group at follow-up (P<0.05). The physical activity level declined in both from baseline to follow-up and the decline was largest in the hyperglycaemic women, with 79% being physically active at baseline and 66% being active at follow-up. The decline was only 5.5% in the normoglycaemic group (**Table 1**).

Spearman's correlation test of BMI and waist circumference, hip circumference and waist-hip ratio at follow-up revealed a significant correlation between the BMI and the other markers of weight (waist: rho=0.84 P<0.0001, hip: rho=0.87 P<0.0001, waist-hip ratio: rho=0.44 P<0.0001) (**Figure 2A-C**),

illustrating that BMI is an acceptable surrogate marker for central obesity in this study population. We found no correlation between BMI and muscle strength (rho=0.08, P=0.009) and no trend in muscle strength profile changes within any of the BMI groups (**Figure 2D**), indicating that subjects with low BMI did not experience reduced muscle strength resulting from low BMI and possible sarcopenic conditions.

BMI change and hyperglycaemia

BMI change after the 13-year follow-up was determined for each baseline BMI group (**Table 2**). Weight-gainers in all BMI groups increased their BMI with two BMI units or more from baseline to follow-up with normalweight women gaining less than the heavier subgroups; 2.5 kg/m^2 [2.3-2.7] gain for normalweight women, 3.1 kg/m^2 [2.8-3.3] for overweight weight-gainers and 4.7 kg/m^2 [2.6-4.1] for obese gainers. Both normalweight and overweight weight-gainers showed to increase BMI to a level now exceeding the BMI of weight-losers from the heavier baseline BMI groups. This exemplified in overweight weight-gainers having a BMI of 30.3 kg/m^2 [30.0-30.6] at follow-up whereas obese weight-losers ended up at a BMI of 28.1 kg/m^2 [27.1-29.0] at follow-up, resulting in a BMI category change for both BMI groups. The same tendency was seen in normal weight-gainers with a follow-up BMI of 25.1 kg/m^2 [24.8-25.4] compared to overweight weight-losers dropping to a follow-up BMI in the normal weight range of 24.4 kg/m^2 [24.1-24.7].

Logistic regression was used to determine the risk of hyperglycaemia in weight-losers, weight-stable, and weight-gainers stratified based on baseline BMI group (**Table 3**). In elderly women who had gained weight at follow-up, regardless of their baseline BMI, we found, in univariate analysis, an increased risk of developing hyperglycaemia in late-life when compared to normalweight women being weight-stable at follow-up. This risk pattern was conserved in both the partly and fully adjusted analysis.

We did observe a differentiated risk as weight-gaining women who were normalweight or overweight at baseline did not increase their risk of hyperglycaemia to a level of that seen in obese weight-gainers (OR=2.5 [1.5-4.0], OR=2.7 [1.6-4.6] and OR=3.2 [1.5-6.6], respectively, in the multivariate analysis). The risk of hyperglycaemia in women with a stable overweight or obese BMI from baseline to follow-

up was 2-fold higher (OR=2.2 [1.3-3.8] and OR=2.0 [1.0-4.21]) than for normalweight women staying normalweight during the follow-up period. The increased risk was not found to be statistically significant in the obese subgroup in the multivariate adjusted analysis, assumedly based on the low number of women in this group (n=46 in the obese group and n=143 in the overweight group) (**Table 3**).

Overweight and obese weight-losers decreased their risk of hyperglycaemia compared to women who were overweight and obese weight-stable after 13 years (**Table 3**).Women who were overweight at baseline and became normalweight during the follow-up period (BMI=24.4 kg/m^2 [24.1-24.7]) showed no clear increased risk of hyperglycaemia, OR=1.1 [0.6-1.9], compared to women who were normalweight at baseline and stayed normalweight at follow-up. The same tendency was also observed for obese weight-losers, OR=1.3 [0.6-2.9], however, as previously highlighted, a lack of power (n=46) could explain the overlapping confidence interval in this subgroup.

Baseline glucose levels, despite being in the normoglycaemic range, were found to have a significant impact on future risk of hyperglycaemia. This was reflected in the multivariate adjusted risk estimates when adjusting for this variable. Further, metabolic risk factors such as triglyceride and systolic blood pressure levels, and current smoking status were found to have an impact on the risk of future hyperglycaemia in elderly women (**Table 3**).

CONCLUSIONS

In this prospective study of weight change and hyperglycaemic risk in elderly women, we found positive effects of weight reduction during a 13-year period, while weight gain increased the risk of hyperglycaemia. Specifically, weight reduction in elderly obese and overweight women showed to decrease their risk of hyperglycaemia in late life, whereas weight gain in both normalweight, overweight, and obese women increased the risk of hyperglycaemia by up to 3-fold when compared to normalweight weight-stable women.

Previous studies focusing on weight change specifically in the elder population have focused mainly on mortality, highlighting how overweight BMI in elderly is associated with a lower relative mortality in this age-group (14–16). Such interpretation can have a drawback by implying that obesity is not as harmful in elderly as compared to middle-aged persons (17). Hyperglycaemic risk, and the development specifically related to obesity is therefore important to monitor in the elderly. This in order to prevent possible development of type 2 diabetes, which can ultimately lead to serious complications, with elderly diabetics having the highest rates of major lower limb amputation (18), myocardial infarction (19), visual impairment (20), and end-stage renal disease (21) of any age-group (8,22).

Our findings suggest that elderly women who gain weight, regardless of baseline BMI level, are at increased risk of hyperglycaemia. This finding is in agreement with previous studies on type 2 diabetes risk emphasizing that weight gain in adulthood is associated with increased risk of developing diabetes (23–26). Further, we showed how weight-loss reduced the risk of hyperglycaemia which is in accordance with reports on decreased incidence of type 2 diabetes among adults aged 60 years or older as a result of weight-loss (27,28). Applying hyperglycaemia as an endpoint, as done in this study, is not directly relatable to a type 2 diabetes diagnosis. However, since hyperglycaemia is the first clinical sign of a higher risk of type 2 diabetes and cardiovascular disease, and since more than half of all older adults suffer from pre-diabetes (9,29,30), the use of hyperglycaemia, as determined by fasting glucose samples, is deemed valid as an initial marker for increased pre-diabetes risk. However, impaired fasting glucose does not reveal the full risk of pre-diabetes. Impaired glucose tolerance, another measure of insulin resistance and risk of pre-diabetes, has not been measured in the PERF study. However, a study performed by Larsson et al. showed in post-menopausal women how the two measures identified different women at risk of pre-diabetes (31).

Several aspects of weight loss need consideration in relation to interpretations of the associated risk of hyperglycaemia in elderly women. In this regard, the use of BMI, as an estimate for weight change, can introduce errors, as fat-free mass (muscle) decreases in elderly while the relative adiposity, and specifically the intra-abdominal fat, continues to increase (32,33), thus changing the proportion of muscle to fat mass in a less healthy direction. Moreover, since height is also reduced with age in women,

BMI calculations can be misleading if continuous measures are lacking. In this cohort, we found no overall change in BMI from baseline to follow-up and this absent change in BMI over a period of 13 years could be masking a loss in muscle mass as height equally decreased. To analyze for a possible bias associated with the use of BMI, we applied correlation analysis between follow-up BMI and waist circumference, hip circumference and waist-hip ratio which correlates highly with both total and intra-abdominal fat (17,34) of which especially the latter plays a vital negative role in metabolic disorders including hyperglycaemia (17). We found a high correlation (r=0.84, r=0.87 and r=0.44, respectively) between follow-up BMI and the additional measurements of abdominal fat, and, contrarily, no pattern of decreased muscle strength as a function of BMI (r=0.08), which supports that BMI can be used as a reliable marker of weight change in this cohort.

We showed how elderly women developing hyperglycaemia in late life (80+ years) had significantly higher fasting glucose levels (still in the normoglycemic range) 13 years prior to revealing a hyperglycemic outcome. This observation of a slightly unhealthier phenotype was further signified as significantly higher weight, systolic blood pressure, triglyceride levels and decreased HDL-cholesterol were present already at baseline. Further, a larger fraction of the women who developed hyperglycaemia at follow-up was smoking. These risk factors have all previously been reported to distort the metabolic state resulting in hypertension, diabetes, and the metabolic syndrome (35). Associating obesity specifically to hyperglycaemia therefore only illustrates part of the dysmetabolic phenotype.

Limitations

Hyperglycaemic women in this study smoke and drink more and exercise less at follow-up. Even though smoking is the only statistically significant observation, it reflects a group of hyperglycaemic women with an assumedly unhealthier lifestyle. We can therefore only speculate whether there are other causal reasons for hyperglycaemia, thus associated with these risk factors, which are confounding the effects of weight gain. This is to some extend tried adjusted for in the multivariate logistic analysis, however, it could be argued that weight change is always a consequence of preceding mediators, thus being a surrogate for a preceding unhealthier phenotype.

In very late life (the 80+ year age group) the prevalence of obesity is approximately half of that in the 50–59-year group (36,37). This overall trend of weight reduction in late life is likely to arise from confounding factors such as survival bias, competing mortalities, smoking, and unintentional weight loss in the sick elderly (17), together with the loss of muscle mass as previously described. Specifically smoking, which is known to induce weight loss in elderly, could distort our results, as the women in our study who develop hyperglycaemia, has a significantly larger fraction of smokers than the women staying normoglycaemic, so losing weight would perhaps reflect smoking status and thereby be associated with being hyperglycaemic.

In this cohort of elderly Caucasian women, we saw an increase in the number of obese women at follow-up. This could indicate that the fraction of elderly women participating in a clinical study in very late life could be somewhat subjective to 'healthy participant's bias', as only the overweight and obese women healthy enough to survive into late-life are included, as opposed to the perhaps weaker obese non-survivors. As a strategy to prevent an even further overrepresentation of a metabolically healthier subgroup in the PERF study, we decided to collect data in the women's own home at follow-up, hence allowing for a broader group of women to be enrolled in the follow-up study.

By means of study design, we sought to eliminate a possible confounding factor by excluding pre-diabetics, women with register-based diabetes diagnosis or reported use of antidiabetic medication from the study group, since this group might curb with the study outcome due to focused weight loss as requested by the general practitioner, thereby introducing reverse causality. However, reverse causation owing to other, either pre-existing or prediabetes related chronic diseases occurring in the follow-up period, may also lead to intentional weight loss, in order to treat the conditions. Possible approaches to addressing these potential biases could be to disregard women with diagnosed cardiovascular and rheumatic diseases, but also cancer, which is known to decrease body weight in elderly, occurring in the follow-up period thereby restricting the analysis to elderly women without any known weight related diseases. However, an introduction of these exclusion criteria would leave in only the most biologically advantaged obese women. Therefore, we decided that this was not the correct approach for this study.

The PERF cohort is a Danish community-based cohort of Caucasian women with tax-paid (not individually paid) health care, and the results obtained in this study would need replication in other ethnic groups and other health care systems to confirm if data are representative for all women. Further, the cohort consists only of women wherefore a similar study in men would be required in order to conclude if weight and weight change would imply the same risk of hyperglycaemia in elderly men.

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CC serves as a board member and stock owner in Nordic Bioscience. MK and KH hold stocks in Nordic Bioscience. KD, JSN, SB, and HBH have nothing to declare.

Author contributions

KD has done the literature search, statistical analysis, figures, data interpretation, and writing. JSN helped with data interpretation and writing, CC was responsible for study design and provided scientific advice, MK helped with data interpretation and scientific advice, SB and KH contributed to the data interpretation, writing and with scientific advice.

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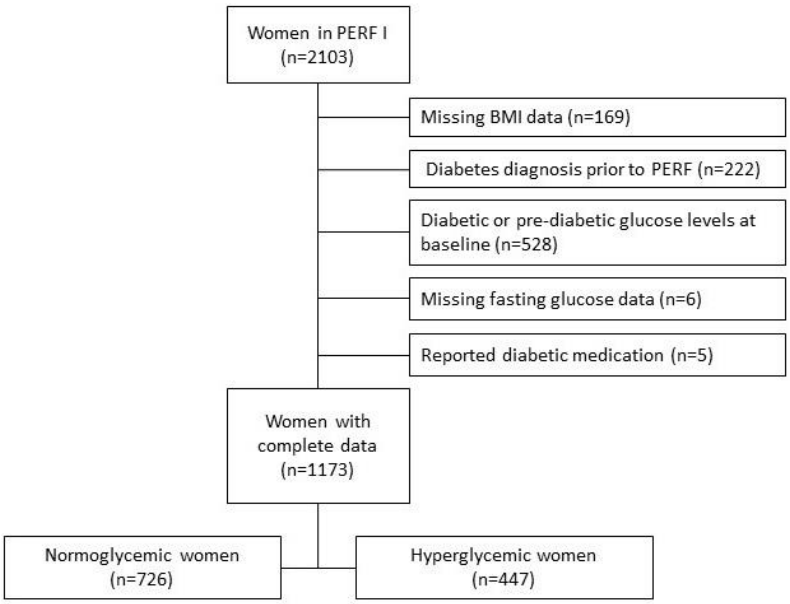

Figure 1. Definition of the study population. Abbreviations: PERF: Prospective Epidemiological Risk Factor study (baseline visit); BMI: Body Mass Index.

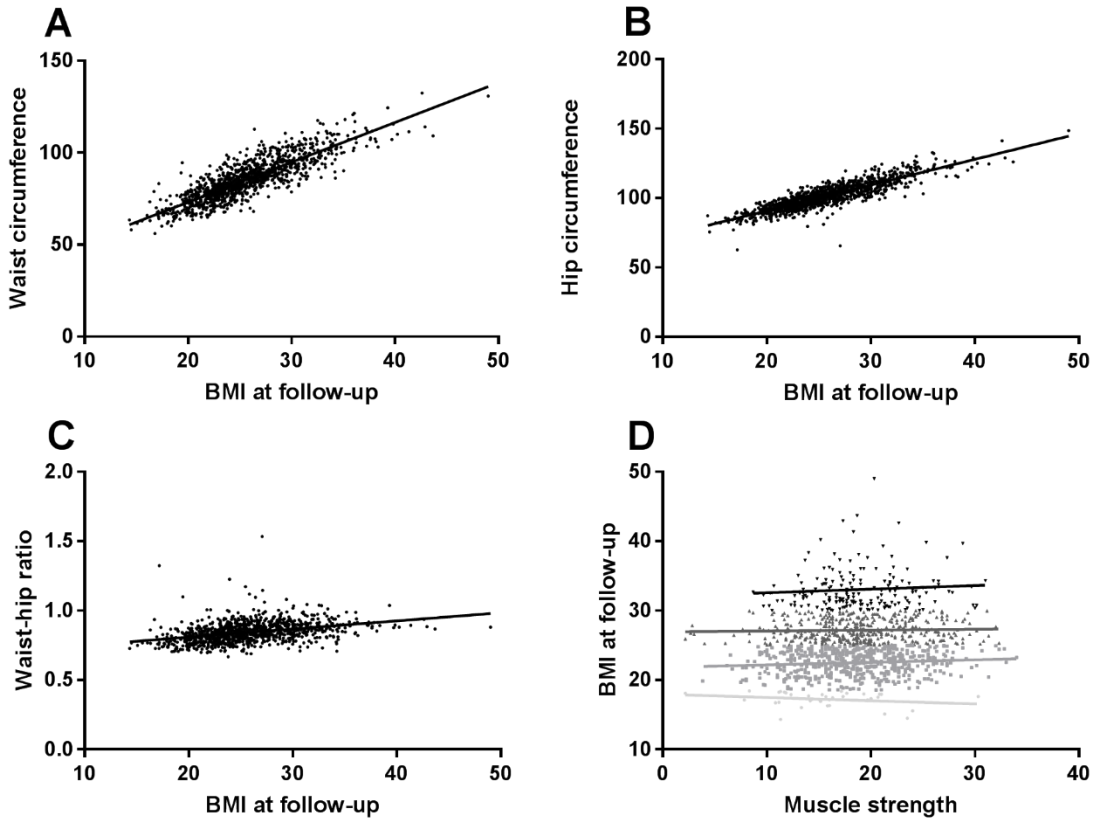

Figure 2. A: Correlation between waist circumference and BMI at follow-up (n=1172). **B:** Correlation between hip circumference and BMI at follow-up (n=1172). **C:** Correlation between waist-hip ratio and BMI at follow-up (n=1172). **D:** Correlation between muscle strength and BMI at follow-up stratified based on BMI group; lightest grey: Underweight at follow-up (n=36), light gray: Normalweight at follow-up (n=551), dark gray: Overweight at follow-up (n=408); black: Obese at follow-up (n=178)

Table 1: Baseline and follow-up information on hyperglycemic and normoglycemic women enrolled in the PERF study (n=1,173). Data are shown as mean value (95% confidence interval) or as percentage (absolute number of cases).

	BASELINE							FOLLOW-UP						
	N	Normoglycemic		N	Future hyperglycaemic		P-value	N	Normoglycemic		N	Hyperglycaemic		p-value
Age (years)	726	67.5	(67.0 – 67.9)	447	67.2	(66.7 – 67.8)	0.6	726	80.8	(80.4 – 81.2)	447	80.6	(80.0 – 81.1)	0.5
Family history of diabetes (%)	634	9.6	(61)	387	9.3	(36)	0.9							
Smoking														
Never (%)	726	54.3	(394)	447	49.7	(222)	0.1	723	51.2	(370)	447	44.3	(198)	0.02
Previous (%)	726	26.4	(192)	447	26.4	(118)	0.99	723	11.6	(84)	447	12.3	(55)	0.7
Current (%)	726	19.3	(140)	447	23.9	(107)	0.06	723	37.2	(269)	447	43.4	(194)	0.04
Alcohol, current (>10.5 units/week, %)	723	40.5	(293)	442	35.1	(155)	0.06	717	0.4	(3)	435	1.1	(5)	0.1
Physical activity (≥2 sessions/week, %)	726	75.5	(548)	447	79.2	(354)	0.1	723	70.0	(506)	444	66.0	(293)	0.2
Height (cm)	726	162.3	(161.9 – 162.7)	447	162.3	(161.8 – 162.9)	0.9	726	160.1	(159.7 – 160.6)	447	160.3	(159.7 – 160.9)	0.7
Weight (kg)	726	66.0	(65.3 – 66.8)	447	68.3	(67.3 – 69.3)	0.0004	726	63.9	(63.1 – 64.8)	447	68.4	(67.2 – 69.6)	<0.0001
BMI (kg/m^2)	726	25.1	(24.8 – 25.3)	447	25.9	(25.6 – 26.3)	0.0002	726	24.9	(24.6 – 25.2)	447	26.6	(26.2 – 27.0)	<0.0001
Underweight (%)	726	1.8	(13)	447	1.3	(6)	0.6	726	3.9	(28)	447	1.8	(8)	0.05
Normal weight (%)	726	55.4	(402)	447	41.8	(187)	<0.0001	726	53.2	(386)	447	36.9	(165)	<0.0001
Overweight (%)	726	32.5	(236)	447	42.7	(191)	0.0004	726	30.7	(223)	447	41.4	(185)	0.0002
Obese (%)	726	10.3	(75)	447	14.1	(63)	0.05	726	12.3	(89)	447	19.9	(89)	0.0004
Systolic blood pressure (mmHg)	726	143.4	(141.8 – 145.1)	447	147.0	(144.9 – 149.1)	0.009	724	139.5	(138.0 – 141.0)	447	141.6	(139.6 – 143.6)	0.09
Diastolic blood pressure (mmHg)	726	81.4	(80.6 – 82.1)	447	81.9	(81.0 – 82.9)	0.4	724	80.2	(79.3 – 81.4)	447	81.6	(80.5 – 82.6)	0.04
Glucose (mmol/L)	726	5.0	(5.0 – 5.0)	447	5.1	(5.1 – 5.2)	<0.0001	726	5.1	(5.0 – 5.1)	447	5.8	(5.8 – 5.9)	<0.0001
Low density lipoprotein (mmol/L)	720	3.8	(3.8 – 3.9)	441	3.9	(3.8 – 4.0)	0.09	726	3.4	(3.3 – 3.4)	447	3.4	(3.2 – 3.5)	0.9
High density lipoprotein (mmol/L)	720	1.8	(1.8 – 1.8)	441	1.7	(1.7 – 1.7)	0.0003	726	2.1	(2.1 – 2.1)	447	1.9	(1.9 – 1.9)	<0.0001
Triglycerides (mmol/L)	726	1.2	(1.2 – 1.2)	446	1.3	(1.3 – 1.4)	<0.0001	726	1.1	(1.1 – 1.2)	447	1.4	(1.3 – 1.4)	<0.0001

Table 2: Baseline body mass index (BMI), BMI change and BMI at follow-up for elderly women in the PERF cohort (n=1,173). Data are shown as mean with 95% confidence interval.

BMI GROUP AT BASELINE	N	BMI AT BASELINE (kg/m2)	BMI CHANGE	N	BMI AT FOLLOW-UP (kg/m2)	ΔBMI (kg/m2)
NORMALWEIGHT	608	22.6 [22.4-22.7]	LOSS	203	20.8 [20.5-21.1]	-1.8 [-2.0-(-)1.7]
			STABLE	202	22.7 [22.4-22.9]	0.2 [0.1-0.3]
			GAIN	203	25.1 [24.8-25.4]	2.5 [2.3-2.7]
OVERWEIGHT	427	27.0 [26.9-27.2]	LOSS	142	24.4 [24.1-24.7]	-2.6 [-2.9-(-)2.3]
			STABLE	143	27.2 [27.0-27.5]	0.4 [0.3-0.5]
			GAIN	142	30.3 [30.0-30.6]	3.1 [2.8-3.3]
OBESE	138	32.6 [32.2-33.0]	LOSS	46	28.1 [27.1-29.0]	-4.7 [-5.3-(-)4.1]
			STABLE	46	32.0 [31.3-32.8]	-0.5 [-0.8-(-)0.2]
			GAIN	46	35.9 [34.8-36.9]	3.7 [2.6-4.1]

Table 3: Univariate and multivariate-adjusted odds ratios (ORs) for risk of hyperglycaemia in normalweight, overweight and obese elderly women with either weight loss, stable weight, or weight gain after 13 years of follow-up (n=1,173). CI: confidence interval.

Variable		Univariate OR (95% CI)	Partly OR (95% CI)	Multivariate OR (95% CI)
Baseline BMI	**BMI Change**			
	Loss	1.16 (0.74-1.80)	1.14 (0.73-1.79)	1.06 (0.63-1.77)
Normal	Stable	1 (reference)	1 (reference)	1 (reference)
	Gain	**2.28 (1.49-3.48)**	**2.31 (1.51-3.55)**	**2.47 (1.52-4.02)**
	Loss	1.41 (0.88-2.27)	1.39 (0.86-2.26)	1.06 (0.61-1.85)
Overweight	Stable	**2.91 (1.84-4.60)**	**2.86 (1.80-4.55)**	**2.22 (1.31-3.75)**
	Gain	**3.50 (2.21-5.54)**	**3.52 (2.22-5.60)**	**2.67 (1.56-4.55)**
	Loss	1.47 (0.73-2.95)	1.44 (0.72-2.91)	1.34 (0.61-2.92)
Obese	Stable	**2.79 (1.44-5.40)**	**2.97 (1.52-5.79)**	2.01 (0.96-4.21)
	Gain	**3.95 (2.03-7.68)**	**4.12 (2.10-8.08)**	**3.20 (1.50-6.80)**
Other Baseline characteristics				
Age (per years of ageing)		1.0 (0.98-1.02)	1.00 (0.98-1.03)	0.98 (0.95-1.00)
Education				
Primary school		1 (reference)	1 (reference)	1 (reference)
High School		0.79 (0.59-1.04)	0.80 (0.60-1.08)	0.81 (0.58-1.14)
University		0.67 (0.43-1.04)	0.71 (0.45-1.12)	0.78 (0.45-1.33)
Smoking history				
Never		1 (reference)	1 (reference)	1 (reference)
Past		1.09 (0.82-1.45)	1.14 (0.85-1.52)	1.20 (0.86-1.67)
Current		1.36 (1.00-1.53)	1.43 (1.04-1.97)	**1.49 (1.04-2.14)**
Alcohol Consumption (>10.5 units/week)		0.80 (0.63-1.02)	0.85 (0.66-1.10)	0.86 (0.65-1.15)
Physical activity (≥2 time/week)		1.23 (0.93-1.64)	1.43 (1.06-1.93)	1.35 (0.97-1.87)
Systolic blood pressure (per 10 mmHg increase)		**1.07 (1.02-1.13)**		**1.11 (1.03-1.21)**
Diastolic blood pressure (per 10 mmHg increase)		1.05 (0.94-1.18)		0.84 (0.71-0.99)
Glucose (mmol/L)		**4.35 (2.94-6.44)**		**4.47 (2.85-7.03)**
Low density lipoprotein (mmol/L)		1.12 (0.98-1.27)		1.04 (0.89-1.21)
High density lipoprotein (mmol/L)		0.58 (0.43-0.78)		0.94 (0.64-1.39)
Triglycerides (mmol/L)		**1.64 (1.30-2.07)**		**1.51 (1.11-2.06)**
Treated hypertension (yes/no)		1.26 (0.92-1.71)		0.97 (0.67-1.42)
Treated dyslipidaemia (yes/no)		1.42 (0.76-2.65)		1.08 (0.52-2.23)
Family history of diabetes		0.96 (0-63-1.49)		1.19 (0.74-1.91)

Additional Results (not published)

Baseline and follow-up characteristics of elderly Caucasian women enrolled in the PERF study (n=1,173) Data shown as mean value (± 95% CI) or as percentage (absolute number of cases).

	N	Baseline		Follow-up		P-value
Age (years)	1173	67.1	(66.8-67.5)	80.5	(80.2-80.8)	-
Family history of diabetes (%)	1021	29.3	(299)		-	-
Smoking, never (%)	1170	52.5	(614)	48.5	(568)	<0.0001
Alcohol, current (≥7gl/week, %)	1144	39.0	(446)	0.7	(8)	<0.0001
Physical activity (≥2 sessions/week, %)	1167	76.9	(897)	68.5	(799)	<0.0001
Height (cm)	1173	162.2	(161.9-162.6)	160.1	(159.7-160.5)	<0.0001
Weight (kg)	1173	66.1	(65.5-66.7)	64.6	(63.9-65.2)	<0.0001
BMI (kg/m^2)	1173	25.1	(24.9-25.3)	25.2	(24.9-25.4)	0.3
Underweight (%)	1173	1.6	(19)	3.1	(36)	0.003
Normal weight (%)	1173	50.2	(589)	47.0	(551)	0.02
Overweight (%)	1173	36.4	(427)	34.8	(408)	0.3
Obese (%)	1173	11.8	(138)	15.2	(178)	0.0005
Systolic blood pressure (mmHg)	1171	142.9	(141.7-144.2)	138.8	(137.6-140.0)	<0.0001
Diastolic blood pressure (mmHg)	1171	80.9	(80.3-81.5)	79.8	(79.1-80.5)	0.008
Glucose (mmol/L)	1173	5.0	(5.0-5.0)	5.3	(5.3-5.4)	<0.0001
Cholesterol (mmol/L)	1173	6.1	(6.0-6.2)	5.6	(5.5-5.6)	<0.0001
Low density lipoprotein (mmol/L)	1161	3.7	(3.7-3.8)	3.2	(3.1-3.3)	<0.0001
High density lipoprotein (mmol/L)	1161	1.7	(1.7-1.7)	2.0	(1.9-2.0)	<0.0001
Triglycerides (mmol/L)	1172	1.2	(1.1-1.2)	1.1	(1.1-1.2)	0.02
White blood cells (10^9cells/L)	1169	5.3	(5.2-5.4)	5.9	(5.8-6.0)	<0.0001
Alanine-aminotransferase (mmol/L)	1173	24.4	(23.9-24.8)	18.7	(18.3-19.1)	<0.0001
Aspartate-aminotransferase (mmol/L)	1173	23.8	(23.5-24.1)	22.8	(22.4-23.1)	<0.0001
Gamma-glutamyltransferase (mmol/L)	1173	30.0	(29.2-30.8)	23.5	(22.6-24.5)	<0.0001

7 COLLAGEN DEGRADATION AND MORTALITY

Title

Matrix Metalloproteinase Mediated Type I Collagen Degradation – An Independent Risk Factor for Mortality in Women.

Aim

The aim of the study was to investigate whether MMP mediated type I collagen degradation (C1M) was predictive of mortality in elderly Danish women.

Rationale

Chronic fibro-proliferative diseases are associated with nearly 45% of all deaths in the developed world. Matrix metalloproteinase (MMP) mediated remodelling of the extracellular matrix (ECM) plays an important role in disease development. Degradation of type I collagen is considered having a major role in this matter.

Findings

Subjects with high serum C1M levels showed significantly increased mortality. The adjusted three-year HR was 2.0 [95% CI: 1.5-2.8] for all-cause mortality, 2.3 [1.5-3.6] for cancer and 1.8 [1.0-3.2] for CVD. The adjusted nine-year HR was 1.50 [1.3-1.8] for all-cause mortality, 1.5 [1.2-1.9] for cancer and 1.7 [1.3-2.2] for CVD.

Conclusions

Increased MMP-mediated tissue degradation, as an independent risk factor, was associated with a 2-fold increase in all-cause mortality within three years of follow-up and a 1.5-fold increase in all-cause mortality up to nine years prior to death.

MMP-mediated tissue degradation may be an important predisposition for the cause of disease and subsequent mortality.

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Original Article

Matrix Metalloproteinase Mediated Type I Collagen Degradation — An Independent Risk Factor for Mortality in Women

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ABSTRACT

Chronic fibro-proliferative diseases are associated with nearly 45% of all deaths in the developed world. Matrix metalloproteinase (MMP) mediated remodeling of the extracellular matrix (ECM) plays an important role in disease development. Degradation of type I collagen is considered having a major role in this matter. C1M is a biomarker measuring type I collagen degradation fragments in blood. The aim of the current study was to investigate whether MMP mediated type I collagen degradation (C1M) was predictive of mortality in a large prospective cohort of Danish women aged 48–89 (n = 5855).

Subjects with high serum C1M showed significant increased mortality. The adjusted three year HR was 2.02 [95% CI: 1.48–2.76] for all-cause mortality, 2.32 [95% CI: 1.51–3.56] for cancer and 1.77 [95% CI: 0.98–3.17] for cardiovascular diseases. The adjusted nine year HR was 1.50 [95% CI: 1.28–1.75] for all-cause mortality, 1.49 [95% CI: 1.16–1.90] for cancer and 1.69 [95% CI: 1.27–2.24] for cardiovascular diseases.

High MMP-mediated type I collagen degradation was associated with increased mortality. Subjects with high C1M had a 2-fold increase in mortality compared to subjects with low levels of this collagen degradation product.

1. Introduction

It is estimated that women in the European (EU15) countries are expected to live approximately 80% of their lives in good health — resulting in a healthy life expectancy up to 20% shorter than their total life expectancy (Health and Consumer Protection Directorate-General, 2006). A major contributor to the decrease in healthy life expectancy is chronic fibroproliferative diseases such as fibrosis and cancer, and nearly 45% of all deaths in the developed world are associated with some form of tissue remodeling disease (Wynn, 2007; Pinzani, 2008). Tissue remodeling in relation to diagnosis and prognosis is therefore a hot topic, consequent to the prevalence of diseases associated with this remodeling, following decreased healthy life expectancy and premature death.

The common denominator of fibroproliferative diseases is dysregulated tissue remodeling causing an accumulation of extracellular matrix (ECM) components in tissues of different organs (Wynn, 2007, 2008; Wynn and Barron, 2010; Schuppan et al., 2001). The ECM consists mainly of collagens, proteoglycans and glycoproteins. Collagens constitute approximately 30% of all proteins in the body, with type I collagen as the most ubiquitous collagen (Muiznieks and Keeley, 2013). Under pathological conditions the normal remodeling balance is disturbed replacing original proteins of the ECM with different matrix components, in turn leading to an altered composition and quality of the matrix (Karsdal et al., 2013a). Emerging evidence suggests that altered components and post-translational modifications (PTMs) of proteins in the ECM may both initiate and drive disease progression (Leeming et al., 2011a).

Matrix metalloproteinases (MMPs) constitute a principal family of enzymes involved in degradation of ECM proteins. The pathological over-expression of MMPs results in small protein fragments holding PTMs, which are released into the blood. These PTM fragments can be referred to as neoepitopes or so-called 'protein fingerprints'. A neoepitope is a protease-generated PTM, which has potential as a biochemical marker of ECM remodeling (Karsdal et al., 2013b). Despite the notion that MMP-mediated ECM remodeling is a central event in initiation and progression of connective tissue diseases (Wynn, 2007; Pinzani, 2008), technologies for measurement are limited in the diagnostic armamentarium.

Type I collagen may be measured by 4 different epitopes (CTX-I, C1M, ICTP and PINP). Measurement of the pro-peptide of type I collagen (PINP) is a standard marker for bone formation, while a cathepsin K degraded product (CTX-I) is the standard measure of bone resorption

Abbreviations: C1M, MMP-mediated type I collagen degradation; CTX-I, Cathepsin K degraded products of C-terminal telopeptides of type I collagen; ECM, Extracellular matrix; MMP, Matrix metalloproteinase; PERF I, Prospective Epidemiological Risk Factor study; PTM, Post-translational modification.

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(Rosenquist et al., 1998). Bone formation and bone resorption are completely different and opposite directed processes, emphasizing the need in clinical chemistry to measure the right protein in the right way. The marker of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP), is an MMP derived intermediate conformational epitope which has been evaluated for ECM-related diseases (Elomaa et al., 1992). Recently we developed an ELISA assay detecting MMP-mediated type I collagen degradation fragments in serum (termed C1M). The competitive ELISA measures the end product of tissue degradation, i.e. a pool of peptides/proteins all having this specific MMP-mediated binding site as the denominator. The monoclonal antibody recognizes a 6 amino acid sequence at position 764 in the C-terminus of type 1 collagen (Leeming et al., 2011b). The collagen degradation fragment is generated by MMPs 2, 9 and 13 and is destroyed by cathepsin K, making this a soft tissue specific marker not originating from bone turnover.

In the period from 1999–2001 a total of 5855 Danish postmenopausal women participated in the large Prospective Epidemiological Risk Factor (PERF I) study aimed at identifying risk factors associated with age-related diseases. Serum samples originally collected in the PERF I cohort were in the current study analyzed in relation to levels of C1M and combined with register data from Danish national registries describing cause and time of death of the deceased part of the cohort.

We hypothesized that MMP-mediated tissue degradation of type I collagen was predictive for mortality.

2. Methods

2.1. Study Design

The Prospective Epidemiologic Risk Factor (PERF I) study was an observational, prospective follow-up study of Danish postmenopausal women who had previously either participated in clinical randomized placebo-controlled studies or were screened without being randomized for previous studies at the Center for Clinical and Basic Research (CCBR) in either Copenhagen or Aalborg. Invitations for participation were done by including all subjects in the CCBR subject database regardless of their previous medical history, ensuring no overrepresentation of subjects with history of specific diseases. A total of 5855 Danish postmenopausal Caucasian women aged 48-89 were enrolled in the PERF I Study from 1999–2001. The study was carried out in accordance with ICH-GCP with study protocol approval from the local ethics committee.

2.2. Baseline Examinations

Vital signs and fasting serum samples were collected at time of enrollment and serum samples were stored at −80 °C for later use. Subjects reported on demographic characteristics; smoking status, alcohol consumption, physical activity and level of education as well as hypertension, hyperlipidemia, cancer history and diabetes in a self-reported questionnaire.

2.3. Outcome Variables

The primary end-points were all-cause mortality and cause specific mortality. Date of death of the deceased sub-group of the PERF I cohort (n = 1505) was obtained from the Danish Civil Registration System and cause of death was obtained from the National Danish Causes of Death Registry. Registry data was obtained up to 31st December 2012 leading to an average follow-up period of 12.1 years (11.4–13.1) for censored subjects. Causes of death were classified according to the International Classification of Diseases, tenth revision (ICD10). The primary cause of death was used for further evaluation of serum C1M levels in specific disease groups; cardiovascular diseases (ICD10 codes I00–I99), cancer (C00–C97), lung diseases (J00–J99), and other deaths (remaining ICD10 codes). Subjects who were dead due to external causes (ICD10 codes V01–X59) were excluded from survival analysis (n = 39). The time of survival was defined as the time from date of enrollment to date of death or to 31st December 2012.

2.4. Type I Collagen Degradation

MMP-degraded type I collagen was measured in serum by enzyme-linked immunosorbent assay (ELISA) as described by Leeming et al. (2011b)(n = 5629). The analyte was tested for stability and was considered to be stable after 12 years of storage (−80 °C). In order to confirm analyte stability, 10 consecutive freeze-thaw cycles were done with no significant change in C1M level. Three year stability studies were performed to validate detection of analyte (C1M), by measuring the same sample in one year intervals. Moreover, the mean level of C1M found in the present study was compared to mean levels of C1M in studies with similar study population conducted at later time points with sample storage of shorter duration.

The cohort was divided into quartiles based on serum C1M level. Q1 (n = 1411): 26.2 ng/mL [21.2–31.3 ng/mL], Q2 (n = 1400): 35.2 ng/mL [31.4–39.5 ng/mL], Q3 (n = 1391): 46.2 ng/mL [39.6–56.0 ng/mL], Q4 (n = 1400): 87.1 ng/mL [56.1–458.8 ng/mL].

Serum CTX-I (n = 5611) was measured by Serum CrossLaps one step ELISA as described by Rosenquist et al. (1998).

2.5. Statistical Analysis

Statistical analysis was conducted using Medcalc® (v 12.3.0) and SAS® (v 9.4). Data are shown as mean ± standard error mean (SEM) if not otherwise indicated. Baseline characteristics of survivors and dead were compared using one-way analysis of variance (ANOVA) for numerical variables while a Chi-square test was used to compare categorical variables (Table 1).

Multivariate Cox proportional-hazard analysis was used to determine proportional hazard ratios for selected risk factors (age, BMI, smoking, exercise, alcohol consumption, education level, hypertension, hyperlipidemia, cancer history and diabetes) (Table 2).

Serum C1M values were normalized using log-transformation. Univariate and multivariate Cox proportional-hazard analysis was used to assess the relation between mortality and serum levels of C1M (log-transformed) in the full follow up period. The adequacy of the Cox proportional-hazard analysis was tested by checking the functional form and the assumption of proportional hazards as described by Lin, Wei, and Ying (Lin et al., 1993). The Kolmogorov-type supremum test revealed no misspecification of the functional forms for the continuous covariates. The proportional hazard assumption was violated with log-transformed C1M in the 12 year follow-up period. Therefore, the multivariate Cox proportional-hazard analysis was split in three year intervals (0–3 years, 3–6 years, 6–9 years, 9–12 years) where the relation between serum levels of C1M (log-transformed) and all-cause mortality was assessed assuming conformity with the proportional hazard assumption in each three year time interval (Fig. 1). Risk factors from Table 2 were included in the multivariate analysis. Likewise the relation between serum levels of CTX-I (log-transformed) and all-cause mortality was assessed in each time interval (data not shown).

Hazard ratios for each quartile of serum C1M was determined by multiplying the parameter estimate of the log-transformed C1M value derived from the multivariate Cox proportional-hazard analysis, with the range between the log-transformed means of serum C1M levels in each quartile, followed by a back-transformation to the original scale using the exponential function. The lower quartile (Q1) was used as reference.

A Kaplan–Meier survival curve was applied to illustrate mortality over time in the four quartiles in the full follow-up period (Fig. 2A) and in three year time intervals (Fig. 2B–E). A log-rank test was used to determine differences between the survival curves.

Table 1
PERF I cohort characteristics. Actual numbers are shown next to percentages.

Variable	Total	Alive	Dead	P-value
	(n = 5855)	(n = 4350)	(n = 1505)	Alive vs. Dead
Age (years)	70.8 ± 0.1	69.4 ± 0.1	74.9 ± 0.2	<0.001
BMI (kg/m^2)	26.2 ± 0.1	26.3 ± 0.1	25.7 ± 0.1	<0.001
Underweight (<18.5) (%)	1.6 (90/5637)	1.1 (46/4226)	3.1 (44/1411)	
Normal (≥18.5–25.0) (%)	41.6 (2343/5637)	40.5 (1713/4226)	44.6 (630/1411)	
Overweight (>25.0–30.0) (%)	39.9 (2248/5637)	40.9 (1729/4226)	36.7 (518/1411)	
Obese (>30.0) (%)	17.0 (956/5637)	17.5 (738/4226)	15.5 (219/1411)	
Current smoking (%)	22.5 (1315/5844)	19.8 (861/4342)	30.2 (454/1502)	<0.0001
Exercise (≥1 time/week, %)	68.5 (4003/5843)	72.9 (3165/4340)	55.8 (838/1503)	<0.0001
Alcohol (≥7 drinks/week, %)	32.6 (1896/5812)	32.7 (1411/4317)	32.4 (485/4795)	0.9
Education (%)				0.03
Primary school	71.5 (4178/5841)	70.6 (3064/4339)	74.2 (1114/1502)	
High school	21.4 (1250/5841)	22.2 (963/4339)	19.1 (287/1502)	
University	7.1 (413/5841)	7.2 (312/4339)	6.7 (101/1502)	
Hypertension (%)	31.0 (1807/5838)	28.9 (1252/4337)	37.0 (555/1501)	<0.0001
Hyperlipidemia (%)	9.1 (530/5845)	9.4 (407/4342)	8.2 (123/1503)	0.2
Cancer history (%)	5.2 (301/5808)	4.1 (178/4313)	8.2 (123/1495)	<0.0001
Diabetes (%)				<0.0001
Type 1	0.7 (39/5845)	0.6 (26/4342)	0.9 (13/1503)	
Type 2	2.4 (144/5845)	1.9 (83/4342)	3.9 (59/1503)	
Serum C1M (ng/mL)	50.7 ± 0.5	49.8 ± 0.6	53.4 ± 1.0	0.001
Serum CTX-I (ng/mL)	0.44 ± 0.003	0.44 ± 0.004	0.44 ± 0.007	0.7

Multivariate Cox proportional-hazard analysis was further used to assess levels of serum C1M (log-transformed) in the time interval from 0–9 years (Fig. 3). Risk factors from Table 2 were included in the multivariate analysis.

Hazard ratios were determined for deaths caused by cancer, cardiovascular diseases, lung diseases and other types of death for subjects with serum C1M level in the upper quartile (Q4) versus subjects in the lower quartile (Q1) in time intervals 0–3 years and 0–9 years (Fig. 4). Hazard ratios for cause-specific diseases were calculated solely on the contribution of deaths with the specific diagnose. The remaining part of the deceased population was excluded from the analysis.

3. Results

3.1. PERF I Cohort Characteristics

Table 1 summarizes baseline characteristics of the PERF I cohort stratified in alive and dead subjects, 12 years after initiation of the study. The mean age for the total population was 70.8 years (49.7–88.8). From study entry until 31st December 2012 a total of 1505 subjects died. The age in the deceased subgroup was significantly higher compared to the group of subjects still alive. The entire cohort was characterized by being slightly overweight (BMI 26.2 ± 0.1) with the deceased subgroup having a significantly lower BMI compared to the subjects still alive (25.7 ± 0.1 versus 26.3 ± 0.1) ($p < 0.001$). The living group was characterized by less smokers (19.8% versus 30.2%), subjects with slightly higher education level (22.2% versus 19.1% high school educated), and a larger proportion of physically active subjects (72.8% versus 55.7%). In the cohort 33% consumed more than 7 drinks/week and the proportion of alcohol-consumers drinking ≥7 drinks/week was equal in the living and the deceased group. The deceased group was characterized by having a significantly higher proportion of hypertensive and diabetic subjects, whereas the proportion of subjects with hyperlipidemia did not differ between the two groups. The proportion of subjects with a history of cancer was significantly larger in the deceased part of the cohort (8.2% versus 4.1%).

The serum C1M level was significantly higher ($p = 0.001$) in the deceased part of the cohort compared to those still alive, while no significant difference was seen in serum levels of CTX-I ($p = 0.7$).

Table 2
Hazard ratios for risk factors associated with mortality.
All hazard ratios are mutually adjusted.

Variable	Multivariate analysis		
	Hazard ratio	95% confidence interval	P-value
Age (years)	1.13	1.12 to 1.14	<0.0001
BMI (kg/m^2)			
Underweight (<18.5)	1.59	1.16 to 2.18	0.004
Normal (≥18.5–25.0)			Reference
Overweight (>25.0–30.0)	0.90	0.80 to 1.02	0.1
Obese (>30.0)	0.86	0.73 to 1.01	0.08
Current smoking (yes/no)	1.90	1.69 to 2.14	<0.0001
Physical inactivity (vs. ≥1 time/week)	1.52	1.36 to 1.70	<0.0001
Alcohol (≥7 drinks/week)	1.07	0.95 to 1.20	0.3
Education			
Primary school			Reference
High school	0.91	0.79 to 1.04	0.2
University	0.90	0.73 to 1.11	0.3
Hypertension (yes/no)	1.18	1.05 to 1.32	0.004
Hyperlipedemia (yes/no)	1.07	0.88 to 1.30	0.5
Cancer history (yes/no)	1.86	1.53 to 2.26	<0.0001
Diabetes (no/Type 1/Type 2)			
Type 1	1.52	0.88 to 2.62	0.1
Type 2	1.88	1.41 to 2.51	<0.0001

3.2. Risk Factors for All-cause Mortality

A multivariate Cox proportional-hazard model was used to assess the independent contribution of risk factors (age, smoking, BMI, physical inactivity, alcohol consumption, education level, hypertension, hyperlipidemia, cancer history and diabetes) to mortality in the cohort (Table 2).

All risk factors, except for education level, alcohol consumption (≥ 7 drinks/week), and hyperlipidemia, were associated with mortality.

3.3. Survival

A multivariate Cox proportional-hazard analysis was used to assess hazard ratios for C1M, after adjusting for risk factors listed in Table 2, in different time intervals from blood sampling until time of death. This was done to determine the predictive nature of C1M (Fig. 1).

An increase in mortality with increasing C1M was observed in the 0–3 year interval. This will introduce a "survivor effect" when applying the three year stratification approach in the remaining time intervals, as it will drive the high risk group towards the low risk group over time.

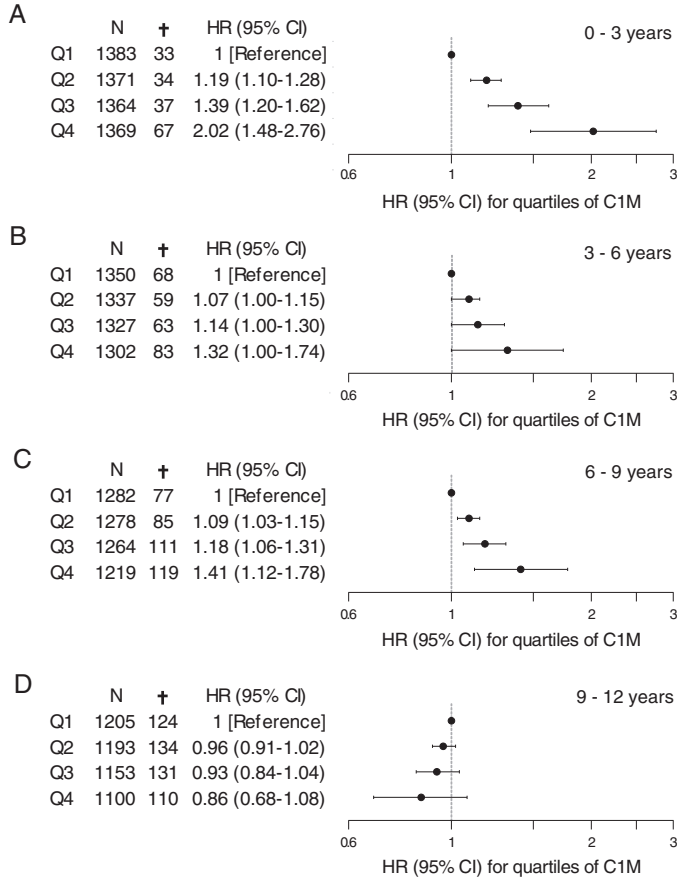

Fig. 1. Hazard ratios with 95% CI for all-cause mortality in quartiles (Q1–Q4) of C1M. A: 0–3, B: 3–6, C: 6–9 and D: 9–12 years. Values are adjusted for age, BMI, smoking, alcohol consumption, physical inactivity, education level, hypertension, hyperlipidemia, cancer history and diabetes.

Despite confounding from "healthy" survivors in the 3–6, 6–9 and 9–12 year intervals, a trend towards an increase in hazard ratio from the lowest quartile (Q1) to the upper quartile (Q4) was observed in all time intervals from 0–9 years. A 2-fold increase in risk of mortality was observed in the interval from 0–3 years (Fig. 1A, HR 2.02 [95% CI: 1.48–2.76]) for subjects with serum C1M levels in the upper quartile (Q4) compared to the lowest quartile (Q1). The same tendency, however non-significant, was seen in the interval from 3–6 years (Fig. 1B, HR 1.32 [95% CI: 1.00–1.74]) and from 6–9 years (Fig. 1C, HR 1.41 [95% CI: 1.12–1.78]). In the interval from 9–12 years no significant change in hazard ratio could be observed between the four quartiles (Fig. 1D).

Contrary, the other type I collagen degradation product (CTX-I) was not found to be a predictor of all cause mortality in neither of the three year intervals from the multivariate Cox proportional-hazard model (data not shown).

A Kaplan–Meier survival curve was applied to illustrate survival over time (Fig. 2A). Part of the cohort with serum C1M levels in the upper quartile (Q4) had a decreased survival probability compared to the three other quartiles in the entire 12 year follow-up period ($p = 0.0001$). No significant difference was seen comparing the lowest quartile (Q1) and the two middle quartiles (Q2 and Q3) in the full follow-up period. Pooled data from Q1–Q3 was used for determining the difference in mortality compared to the upper quartile (Q4). A significant difference in mortality was seen in time intervals from 0–9 years; 0–3 years ($p = 0.0001$), 3–6 years ($p = 0.01$), and 6–9 years ($p = 0.002$) (Fig. 2B–D). No difference in mortality was found in the time interval from 9–12 years ($p = 0.38$, Fig. 2E).

A Cox proportional-hazard analysis was used to assess the mortality risk in part of the follow-up period where an increase in hazard ratio was observed (0–9 year) (Fig. 3). In the univariate Cox proportional-hazard analysis a 59% increased risk of mortality (HR 1.59 [95% CI: 1.38–1.85]) was found, when comparing Q4 to Q1 (Fig. 3A). The increase in mortality risk was 50% (HR 1.50 [95% CI: 1.28–1.75]) within the nine year follow-up period when a multivariate Cox proportional-hazard analysis was applied accounting for risk factors known to impact mortality (Fig. 3B).

3.4. Cause Specific Mortality

Cause specific mortality was assessed in part of the cohort with serum C1M levels in the upper quartile (Q4) versus subjects with serum C1M levels in the lowest quartile (Q1). A multivariate Cox proportional-hazard analysis was used to assess the cause specific mortality risk in the time intervals 0–3 years and 0–9 years (Fig. 4).

In the multivariate Cox regression-analysis, the hazard ratio was 2.32 [95% CI: 1.51–3.56] for cancer, 1.77 [95% CI: 0.98–3.17] for cardiovascular diseases, 1.67 [95% CI: 0.48–5.84] for lung diseases, and 1.77 [95% CI: 0.71–4.42] for other deaths within the 0–3 year interval (Fig. 4A).

In the 0–9 year follow-up interval, the hazard ratio was 1.49 [95% CI: 1.16–1.90] for cancer, 1.69 [95% CI: 1.27–2.24] for cardiovascular diseases, 1.09 [95% CI: 0.63–1.88] for lung diseases, and 1.63 [95% CI: 1.19–2.24] for other deaths (Fig. 4B).

4. Discussion

We have identified MMP-mediated type I collagen degradation (C1M) as an independent risk factor for all-cause mortality. Contrary, we found no association between cathepsin K degraded type I collagen (CTX-I) and all-cause mortality. This suggests that specifically MMP-mediated tissue degradation of type I collagen is associated with mortality.

We found a 2-fold increase in mortality risk in the first three years of follow-up and a 1.5-fold increase was observed with nine year follow-up time in individuals having high MMP-mediated type I collagen degradation compared to individuals with a low serum level of this type I collagen degradation marker.

During pathological remodeling of the ECM excessive levels of tissue- and pathology-specific turnover products are released into the circulation consequently becoming biomarkers. In the present study degradation of type I collagen was measured as a marker for tissue degradation as it is assumed to be a key player in ECM remodeling. Our results emphasize that the enzymatic processing is important since only the MMP-mediated type I collagen degradation was predictive of mortality, not cathepsin K degraded type I collagen. Increased serum C1M levels have previously been shown to be associated with diseases in which chronic inflammation is a key driver, such as ankylosing spondylitis (Bay-Jensen et al., 2012), osteoarthritis (Siebuhr et al., 2014), rheumatoid arthritis (Bay-Jensen et al., 2014), and different types of fibrosis (Leeming et al., 2012, 2013)- diseases which are all contributing to a decreased healthy life expectancy and ultimately death.

The prognostic nature of C1M was assessed by dividing the follow-up period into three year intervals. A 2-fold increase in risk of mortality was determined within the first three years of the follow up period. The increase in mortality with increasing C1M observed in the 0–3 year interval introduce a "survivor effect" when applying the three year stratification approach. This may explain why the HRs decrease over time, driving the associations towards the null hypothesis in the remaining time intervals (3–12 years). The observed potential association in the intermediate time spans (3–6 and 6–9 years) is therefore very likely, but presumably underestimated since the "survivor effect" will drive the high risk group towards the low risk group over time. Despite this, we believe that C1M may predict an increased risk of mortality up to nine years prior to death for subjects with C1M levels in the upper quartile. A 1.5-fold hazard ratio was determined for the combined 0–9 year interval (Fig. 3). The higher risk in the 0–3 year interval underlines the understanding that an event, in this case death, is easier to

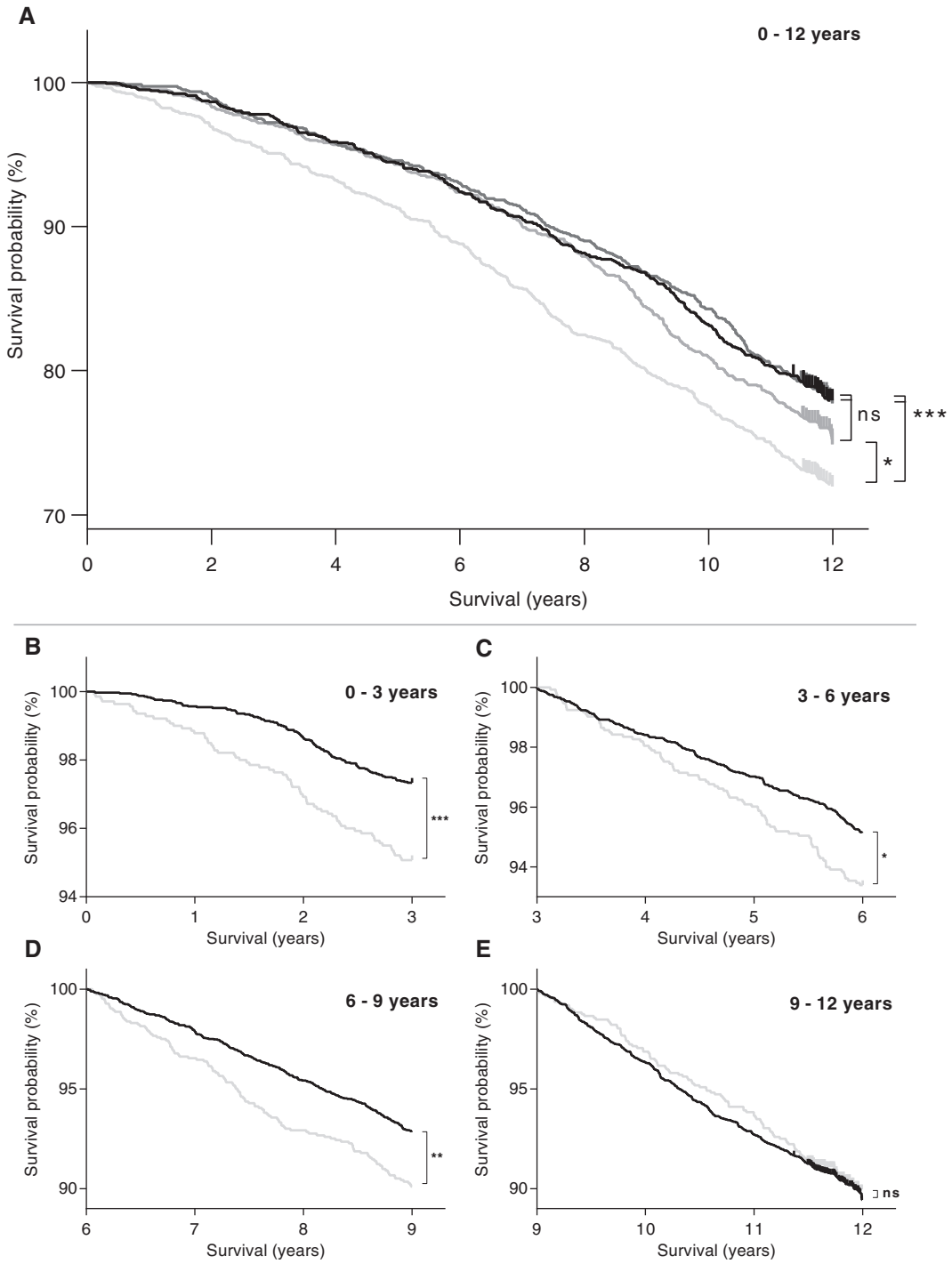

Fig. 2. Kaplan–Meier survival curves for A: 0–12 years with C1M levels divided into quartiles (Q1 (lowest), Q2, Q3 and Q4). Black: Q1, dark gray: Q2, gray: Q3, light gray: Q4; B: 0–3 years, C: 3–6 years, D: 6–9 years and E: 9–12 years with C1M levels divided into Q1–Q3 (pooled) and Q4 (upper quartile). Black: Q1–Q3, light gray: Q4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant.

predict closer to time of occurrence. Subjects with high MMP-mediated type I collagen degradation may therefore be predisposed to a decreased life expectancy based solely on their degree of type I collagen degradation.

The PERF I cohort comprised slightly overweight elderly women at risk of developing common western-lifestyle diseases such as type II diabetes, hypertension and hyperlipidemia. These lifestyle diseases affect many tissues and organs resulting in chronic low grade inflammation possibly following fibroproliferative changes to the ECM and thereby collagen degradation. The most prevalent primary causes of death in the PERF cohort were cancer and cardiovascular diseases accounting for 34% and 27% of all deaths, respectively. Similarly, the two largest causes of death for women aged 70–74 in the EU, as reported in the European Health Report, are cancer and cardiovascular diseases accounting for 37% and 42% respectively (WHO, 2013). High MMP-mediated type I collagen degradation was associated with both cancer and cardiovascular mortality. At first glance, two markedly different diseases, however with increased tissue turnover being a common denominator of both diseases. The risk of dying from cancer was increased 2.3-fold in the first three years of follow-up and an approximate 1.5-fold

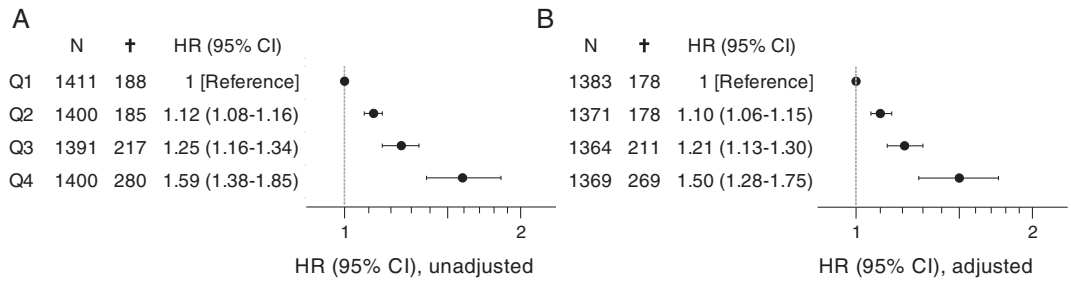

Fig. 3. Hazard ratios with 95% CI for all-cause mortality in quartiles (Q1–Q4) of C1M with nine year follow-up. A: unadjusted, B: adjusted. Adjusted values are corrected for age, BMI, smoking, alcohol consumption, physical inactivity, education level, hypertension, hyperlipidemia, cancer history and diabetes.

increase was observed within the nine year follow-up period in individuals having high MMP-mediated type I collagen degradation. These findings correspond well with the association between ECM remodeling and tumorgenesis (Bonnans et al., 2014; Lu et al., 2012) as ECM remodeling in cancer leads to a dysregulation in tumor growth, inflammation, tissue invasion, and metastasis (Kessenbrock et al., 2010).

In addition, risk of dying from cardiovascular diseases was increased 1.8-fold in the first three years of follow-up and an approximate 1.7-fold increase was observed with nine year follow-up period in individuals having high MMP-mediated type I collagen degradation. Atherosclerosis is a typical hallmark of cardiovascular diseases leading to a disturbance of the ECM homeostasis in the artery wall combined with low-grade inflammation. This results in a disrupted structure of the ECM of the artery wall, ultimately leading to cardiovascular disease and fatal events (Hobeika et al., 2007; Raines, 2000; Galis and Khatri, 2002). Other tissue turnover markers have been associated with mortality; albeit not type I collagen degradation by MMPs. P3NP, a formation marker of type III collagen, was associated with all-cause mortality in the Framingham study (Velagaleti et al., 2010). Endostatin, a degradation fragment of type XVIII collagen, was associated with all-cause, cancer and cardiovascular mortality in two independent cohorts from Sweden (Ärnlöv et al., 2013).

Degradation and formation are interlinked in the tissue turnover balance, making both processes equally important. Determining the better biomarker is therefore not easy. Formation markers, like P3NP, are generated in all tissues comprising type III collagen. However, when measuring a MMP-mediated degradation product, like C1M, it is a prerequisite that the protease is co-expressed in the affected tissue, making this a specific marker for pathologic tissue turnover. When assessing mortality, MMP-mediated type I collagen degradation may possibly either reflect a consequence or a cause of disease leading to mortality (Karsdal et al., 2010). In order to further answer this question, it would be beneficial to have sequential measurements of C1M which could more closely relate diagnosis of disease rather than early prognosis. In the current study it can only be speculated that some individuals may be predisposed for an increased degradation, potentially making them prone to certain diseases and eventually premature death.

Increased serum levels of C1M have shown to be associated with pain and progression of disease in rheumatoid arthritis, and conversely, a decrease by anti-inflammatory modulation (anti-interleukin-6) of more than 35% was associated with protection from disease progression (Siebuhr et al., 2013). This may suggest that attenuation of high remodeling by intervention could be associated with increased life-span. The relation between inflammation and tissue turnover is of particular interest. In autoimmune diseases like rheumatoid arthritis CRP and C1M have been proven to be highly correlated (Siebuhr et al., 2013). In diseases like fibrosis, inflammation may initiate disease, however once present fibrosis can progress without inflammation (Trautwein et al., 2015). The nature and extent of inflammation and ECM remodeling are therefore likely to be very different in different diseases and stages within the same disease. Although this current study identified the

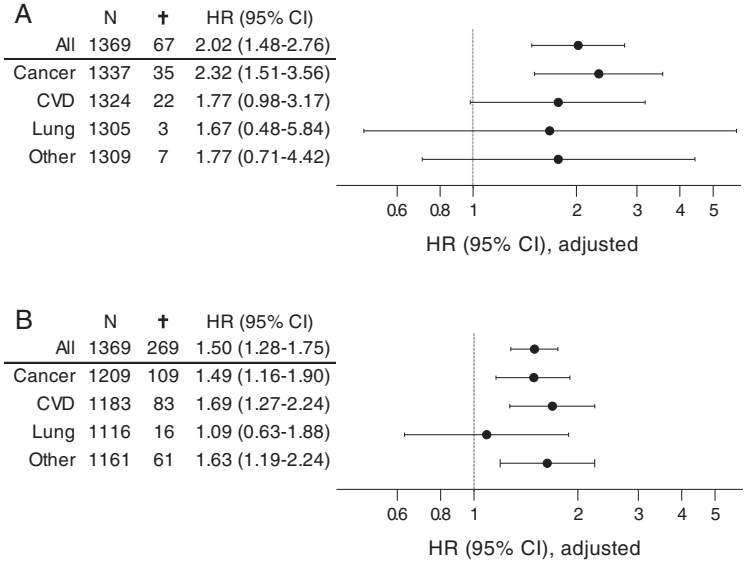

Fig. 4. Hazard ratios with 95% CI for all-cause mortality and cause specific mortality (cancer, cardiovascular diseases, lung diseases and other diseases) for the upper quartile of the cohort (Q4) in time intervals 0–3 years (A) and 0–9 years (A). Hazard ratios are adjusted for age, BMI, smoking, alcohol consumption, physical inactivity, education level, hypertension, hyperlipidemia, cancer history and diabetes.

prognostic importance of C1M assessment in serum, it remains to be shown whether lowering this marker can result in a reduction of the mortality risk.

Interpreting biochemical markers found in serum is associated with many limitations, as several different tissues at different rates may produce and thus contribute to the total pool of molecular marker. Type I collagen is highly abundant in many tissues throughout the body, and an increase in the serological levels of C1M is a hallmark of several fibroproliferative diseases. Further studies on disease-specific contributions to the total pool of ECM remodeling are therefore needed. Importantly however, measuring increased levels may assist in identifying the sub-groups predisposed for increased ECM remodeling. This could aid in early diagnosis of subjects with high tissue turnover, leading to connective tissue diseases, which may benefit from increased medical attention thereby potentially increasing their lifespan.

This cohort is solely comprised of Danish postmenopausal women and further generalization to other demographics needs to be investigated. However, the risk factors identified in the Cox proportional-hazard analysis (smoking, alcohol consumption, physical inactivity, education level, hypertension, hyperlipidemia and diabetes) had similar associations to risk factors found in the Nurses' Health Study, a cohort of middle-aged women (Baer et al., 2011).

Moreover, as in other epidemiological studies, findings in the present study may be affected by selection bias caused by possible over-representation of relatively healthy subjects in the cohort. One could however argue that this would tend to draw the results in a direction towards the null hypothesis and therefore cannot explain our positive results.

5. Conclusion

We found that increased MMP-mediated tissue degradation, as an independent risk factor, was associated with a 2-fold increase in all-cause mortality within three years of follow-up and a 1.5-fold increase in all-cause mortality up to nine years prior to death.

MMP-mediated tissue degradation may be an important predisposition for cause of disease and subsequent mortality.

Author contributions

Katrine Dragsbæk and Jesper Skov Neergaard: writing, literature search, figures, data and statistical analysis, data interpretation.

Henrik Bo Hansen: data interpretation.

Inger Byrjalsen: statistical analysis, data interpretation.

Stephanie Nina Kehlet: sample analysis, data interpretation.

Anne-Christine Bay-Jensen: writing, sample analysis and stability

Peter Alexandersen and Claus Christiansen: study design, scientific advice.

Morten Karsdal: writing, data interpretation, scientific advice.

Competing interests

Anne-Christine Bay-Jensen, Morten Karsdal and Claus Christiansen are stock owners of Nordic Bioscience.

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8 GENERAL DISCUSSION

Longitudinal studies can aid in identifying the predictors of disease, which can then be assessed and ultimately manipulated for their determinant roles in reducing the functional decline in ageing persons (149). The work presented in this thesis manifests the importance of studying ageing and age-related diseases in a longitudinal set-up. We clearly describe how some elderly women are of greater risk of disease and death than others, as a result of specific risk factors contributing to distortion of the healthy ageing process. The description of these risk factors has contributed specifically to broadening the knowledge of non-communicable diseases and death in elderly Danish women.

The PERF study is one of the largest longitudinal ageing studies focusing specifically on women's health. Other longitudinal studies with specific focus on female health are the Women's Health and Aging Study (WHAS I and II) (185), the Study of Women's Health Across the Nation (SWAN) (150), and the Nurses' Health Study (NHS) (186), all from the United States, together with The Australian Longitudinal Study on Women's Health (151). The PERF study is thus believed to contribute to this list of female ageing studies by specifically providing knowledge about Danish female health in ageing.

8.1 Ageing and Tissue Degradation

The WHO describes the complexity of the ageing process in simple terms (11):

'The changes that constitute and influence ageing are complex. At a biological level, ageing is associated with the gradual accumulation of a wide variety of molecular and cellular damage. Over time, this damage leads to a gradual decrease in physiological reserves, an increased risk of many diseases, and a general decline in the capacity of the individual. Ultimately, it will result in death.'

We add further evidence to this statement with the study of collagen type I degradation and how an increased degradation leads to premature death. This finding underlines how some women age better than others and that the risk of mortality is related to MMP-mediated tissue degradation of collagen type I.

Collagen turnover is highly affected by age wherefore the study of degraded collagen can describe different outcomes in different populations (152). The PERF study thus provides

a solid cohort for the study of collagen type I degradation specifically in relation to ageing as described in study IV. Previous studies in the same cohort have added significantly to the knowledge about collagen degradation consequent to the loss of sex hormones, resulting in a corresponding loss in bone mineral density followed by bone resorption (187, 188). However, with the application of C1M, a marker specifically related to tissue degradation *not* associated with bone loss (144,153), it adds to the understanding of underlying cellular damage of connective tissue, the extracellular matrix as a scaffold, and how this is associated with the ageing process.

We found that women with the highest levels of the degradation marker C1M presented an independent risk of premature mortality compared to women with lower levels of this tissue degradation marker. This is assumed to reflect that the extracellular matrix composition and quality may very well be an important predisposition for disease and ultimately death. However, the argument of what triggers excessive tissue degradation is still debatable; or put differently: what comes first? Either, the influence of a risk factor, e.g. excess adipose tissue, which thereby triggers the altering of the tissue homeostasis leading to excessive tissue degradation. Or, the other way around, an underlying genetic predisposition of a high level of tissue degradation leads to an increased adverse impact of other risk factors? The multivariate statistical analysis performed in the study reflects an independent contribution of the marker to the increased risk of mortality. However, it does not reveal if there is an underlying impact, or triggering effect, from other risk factors, as in the scenario described previously.

Siebuhr et al. investigated whether serum levels of C1M could aid in the identification of rheumatoid arthritis patients with fast progression of disease and whether C1M levels were altered following treatment of the disease (154). They found how high levels of C1M at baseline was a significant predictor of disease progression and that treatment dose-dependently lowered the serum level of C1M. This study does not give us the answer to what triggers the inflammatory development of rheumatoid arthritis, but it does however reveal that the extent of MMP-mediated collagen type 1 degradation is modifiable by medical treatment of the inflammatory processes. Applying this association in the setting of the ageing population, a population described by higher levels of low-grade chronic inflammation, it can be speculated if what is presented in study IV as MMP-mediated tissue degradation is a somewhat surrogate or perhaps the result of the low-inflammatory state. It is evident, that especially monocytes and macrophages, are the most important mediators of chronic inflammation with functions described to have a direct effect on toxicity of the

extracellular matrix and collagen deposition (155). Future studies, in the PERF cohort, correlating levels of pro-inflammatory markers, triggered by the mononuclear phagocyte system, with levels of C1M, would be interesting and aid in the understanding of the low-grade inflammatory state associated with ageing and following tissue degradation.

8.2 Metabolic Risk Factors Distorting Healthy Ageing

From a clinical point of view, the presented statement from the WHO can be translated into the conclusion that most major health problems surface during the late years of life resulting in an increased risk of age-related diseases.

The associations between risk factors and following disease are described in study II, highlighting how women with metabolic risk factors presented a greater risk of developing T2DM and CVD.

Insulin resistance forms an underlying 'common ground', as presented in figure 1 of the introduction, for the development of both hyperglycaemia, hypertension and dyslipidaemia, ultimately leading to T2DM and CVD. Insulin resistance is often, though not always, originating from excess adiposity and abdominal obesity. It is therefore generally accepted that insulin resistance and abdominal obesity both are key features in the pathophysiology of these outcomes (57). This is well reflected in study II where we find obesity to be the only risk factor, of the MetS, associated with increased risk of both T2DM and CVD.

8.3 Relevance of the Metabolic Syndrome

The obvious need for a research instrument in the presentation of the combination of risk factors commonly associating with T2DM and CVD has resulted in the development of several different clinical definitions of the MetS, as highlighted in table 1. The concept of the MetS has been, and still is, a topic of great debate, especially concerning its pathogenesis and clinical usefulness (156–158).

The term MetS is not new and extensive research has been undertaken to define its epidemiology. However, studies conducted solely in the ageing population are still sparse. Especially when taking into account the many definitions which are somewhat difficult to compare.

The main differences in the agreement of the usefulness of applying a syndrome-definition are caused by different visions (from the WHO definition to NCEP-ATPIII), of whether or not to specifically demonstrate insulin resistance or instead apply the surrogate measure of obesity (159). Nevertheless, and regardless of the chosen definition, the concept of focusing on multiple risk factors concurrently improves the understanding of the underlying pathophysiology and the mechanisms by which metabolic risk factors like hypertension, dyslipidaemia and hyperglycaemia are interlinked as a result of insulin resistance. It further provides insights into the mechanisms by which obesity influence both insulin target tissues and vasculature, thereby improving the overall understanding of the link between obesity, T2DM, and CVD.

With the definition introduced by the IDF, which is applied in the current work, the thresholds for increased waist circumference and glucose levels were lowered compared to previous definitions. In concept, this new definition, as a thought construct, has advantages because it elegantly puts forward the central role of abdominal obesity in the MetS. However, we showed, that applying this construct, at least in an ageing cohort, leaves out a subgroup not meeting the threshold. The subgroup thus still provided significantly increased risk of disease by holding other risk factors also known to manifest in T2DM and CVD. We therefore argue, that the application of the MetS, thus cannot be used to describe a synergistic effect associated with the clustering of risk factors, when demands for a set pre-requisite, assuming central obesity to always manifest, in order for the risk factors to be interlinked. This is further backed up by studies showing that the incidence of T2DM is higher among adults with normal weight but with insulin resistance or MetS, defined by the NCEP-ATPIII, where central obesity is not a pre-requisite than in overweight subjects who do not have insulin resistance or the MetS (160,161). This gives rise to the discussion of why the MetS is defined, and what it is used for. Is it applied based on evidence of a synergistic risk occurring when requirements for the MetS are fulfilled (those requirements being continuously changed during the last decade) or is it merely a clinical construct used for clinicians to comprehend the common clustering of metabolic risk factors associated with the increasing diagnosis of non-communicable diseases? Regardless of the rationale, the application of the definition aids in the focus of treating multiple risk factors simultaneously. However, we do argue to apply a construct that values the amount of presented risk factors higher than the impact of a specified pre-requisite.

8.3.1 Risk prediction in elderly

The risk prediction in the PERF cohort is stronger in regards to T2DM outcome than CVD risk. Ford et al. confirm this observation in their quantitative review from 2008 of prospective studies examining the association between the MetS and incident diabetes. They conclude that the MetS predicts diabetes more strongly than it predicts coronary heart disease events. Although considerable heterogeneity existed among the studies in the review, as a result of the many MetS definitions, the average estimated relative risk of 3.5–5.2 for incident diabetes with any MetS criteria is greater than the association of MetS with CVD events, with relative risks from 1.5 to 2.0 (105,162). These data confirm the MetS to be more strongly associated with risk for incident diabetes, concluding how this most like relates to particular fasting glucose and waist circumference as they are more strongly associated with diabetes risk than with CVD (163). This is confirmed in the PERF cohort set-up, where we similarly see a strong association specifically between elevated FPG, central obesity and risk of T2DM. This association was not present in the analysis of risk factor contributions for a CVD outcome, where hypertension and central obesity were the main components driving the outcome (figure 2, study II). If the underlying risk factors, although commonly believed to associate from a 'common ground' of insulin resistance, is not to a greater extent overlapping than what is observed in the PERF study, it could be argued that risk scores specifically focusing on one or the other outcome would aid in a more specific risk prediction of the specific outcome.

A variety of both diabetes and CVD risk prediction models have already been proposed and will not be further elaborated upon in this thesis. However, one is worth mentioning; The Diabetes Risk Score, as this score has been compared to the MetS in three different studies (157,164,165). The Diabetes Risk Score includes age, sex, ethnicity, fasting glucose, systolic blood pressure, HDL cholesterol, BMI and family history of diabetes (166). These components are highly comparable to the MetS criteria, however with the great difference, that they are assessed in a continuous manner. Contrary to this risk estimation approach, the application of the MetS, as well as its component risk factors, is entirely dependent on the choice of cut-points to dichotomize the population into those with and without the condition. Dichotomizing continuous variables results in a loss of predictive power, which is confirmed in the three studies, where the risk of diabetes was significantly larger using The Diabetes Risk Score, than what was predicted using the MetS defined cut-points. Assessment of the MetS, using continuous variables, have been performed by Wijndaele et al. in 2006 with CVD as outcome variable. First, they describe, how PCA was applied to the

normalized risk factors in order to extract PCs representing large fractions of MetS variance, revealing two PCs with eigenvalue ≥1. Second, a continuous MetS score was calculated by summing both PC scores, each weighted for the relative contribution of PC1 and PC2 in the described variance. Using this approach, they start by confirming that the continuous MetS score is found to be significantly higher in part of the cohort originally diagnosed with MetS using the IDF definition and that the continuous MetS risk increased progressively with increasing numbers of risk factors. They conclude, that the continuous score is a more suitable and valid alternative for epidemiological analyses, although the binary definition remains useful for clinical practice (167). It would be interesting, to apply this approach to the PERF dataset using the PCA setup. This would aid in the understanding of the different sub-groups not defined with the syndrome and how the risk would increase with increasing numbers of risk factors in all of the defined groups.

8.3.2 Metabolic risk in elderly

The currently available definitions of the MetS do not specifically consider the effect of ageing on the five diagnostic variables. In a study performed on the Italian Longitudinal Study of Aging, the diagnosis of MetS according to the IDF criteria showed no relationship to the risk of myocardial infarction or stroke in elderly without a previous CVD diagnosis (168). In study II we do find an increased risk of a CVD diagnosis, in women with no previous CVD diagnosis. Applying a clinical construct, as the MetS, in a cohort like the PERF cohort, will exclude a large fraction of the women based on the exclusion related to CVD outcomes prior to the initial visit (PERF I). However, as it has been shown that patients with clinical manifestations of atherosclerosis are generally considered to have more than 20% absolute risk of developing a new CVD events in the next 10 years (169), this would indicate an even higher risk in this particular group, most likely associated with the MetS defining risk factors. It would consequently be interesting to conduct the same analysis as described in study II, but on the previously diagnosed CVD subjects, excluded from the analysis, in order to determine whether risk predictions would vary in the two groups. This would aid in understanding if the syndrome is able to discriminate future risk of an outcome based on preceding disease.

8.3.3 The age-dependent metabolic syndrome

Another aspect of the syndrome not being age focused is the missing application of age as part of the criteria. The waist circumference criterion is modified in the IDF definition based on ethnicity and sex, however, cut-off values are not modified according to age. None of the classifications described in Table 1 in the introduction have considered the age-dependent effects, even though a significant age-dependent increase of several parameters has been shown (189-192).

The worldwide increase in the ageing population, combined with the increased prevalence of obesity, are likely to result in the increasing prevalence of MetS reported in recent years. The question is whether the increased metabolic parameters seen in the elderly should be considered as consequent risk factors for disease or natural age-dependent phenomena. Figure 2 in study II reflect, how none of the lipid parameters of the syndrome contributed to the increased risk of neither T2DM nor CVD, questioning if they should be taken into consideration particularly in the diagnosis of MetS in elderly. Put simply, it should be decided whether to apply the same criteria in the diagnosis of MetS for the elderly, as done in the middle-aged population. Limited research has been conducted on this topic, however, Motta et al. have in 2008 studied the relevance of an age-specific definition, employing age-specific cut-points, and how this compared to the IDF criteria in predicting vascular complications. They conclude that the IDF-criterion is not useful for diagnosing MetS in the elderly patients and further highlighting the need of further epidemiological studies of long duration, and wide coverage, involving numerous correlated parameters, in relation with the MetS, age, and ethnicity of the patients. An interesting observation done by the group was further to calculate the prevalence of acute myocardium infarct and stroke in elderly with and without a waist circumference above what is proposed as the entrance criteria for the IDF definition. They found no difference in the subjects with and without central obesity, arguing how this explains the diversity of many proposals, which are often contradictory, on the risk of CVD. These data do indicate that the cut-points of the five set IDF risk factors are not valid for the diagnosis of MetS in the elderly, and it would be interesting to verify this use of age-specific cut-points in the PERF cohort in order to determine if the model proposed by Motta et al. is equally predicting the age-related risk in a cohort in which the age-specific definition was not developed.

8.4 Weight and Weight Change in Late-life

Although obesity is a risk factor for morbidity and mortality in the younger part of the population, the effect of obesity in elderly is much more complex (170). We confirm in study III how weight gain increases the risk of hyperglycaemia in overweight and obese elderly women compared to those classified as normalweight at baseline. We thereby underline that a significant risk of hyperglycaemia is linked to obesity in elderly women.

Elderly women with high levels of body fat who do not appear overweight as a result of loss of lean tissue, manifested as sarcopenic obesity, are believed to be somewhat limited within the PERF cohort, as we see no tendencies in decreasing muscle mass within any of the BMI groups (figure 3, study III). Further, as correlations between BMI and waist-hip-circumference were found to show acceptable correlation, it is argued that BMI is a valid tool for the assessment of weight in the PERF cohort. However, the age-related height modification, which can obstruct BMI measurement, was clearly present in the PERF cohort (see additional table in study III). This phenomenon has previously been reported to induce a false BMI increase of 2.5 units in women suggesting an overweight BMI in elderly to instead be defined as a BMI ≥27.0 (171). This approach of an age-dependent BMI threshold runs well in line with the above discussion related to an age-dependent MetS criteria in elderly. However, in the presented study III in which BMI is used, the weight definition merely functions as a tool for division of the women in the cohort. The cut-points can be looked upon as being somewhat arbitrary, as long as they divide the women in baseline BMI groups, which reflect their weight in a clear manner. However, it would be interesting to run the statistical analysis with this age-dependent cut-point for BMI in order to see if risk estimates in the overweight and obese group would change.

In relation to weight change, evidence from previous studies underlines how body weight associated with maximal survival increases with increasing age (170). This is by Chapman referred to as the 'obesity paradox', as increased weight leads to increased CVD morbidity but a decrease in CVD-related mortality (170). Chapman therefore argues, that there are few if any indications for recommending weight loss to older people based on their weight alone (170). However, *intentional* weight loss by obese elderly is likely to be beneficial if they have obesity-related morbidities and Harrington et al. confirms in a fairly recent meta-analysis how intentional weight loss is not related to increased mortality (172). In study III the reason for weight loss is not known, as we merely work with non-interventional assessment of the cohort, wherefore we cannot argue if it is intentional or not. However, a

clear association is observed between lowered weight and decreased risk of hyperglycaemia. Other studies regarding weight loss specifically in elderly are greatly focused upon the evidence that weight loss by overweight older people is associated with improved quality of life. In the Nurses' Health Study, weight loss in initially overweight women was associated with improved physical function and vitality as well as decreased pain (173). Overall, weight change and its relation to risk factor development in longitudinal setups are sparse. The current literature either focus on weight as the main component of the conducted research or the associated risk factor, e.g. hyperglycaemia, as the component of greatest importance for the health outcomes. The combination of the two associations is therefore somewhat limited, which clearly justifies the need for the research presented in study III.

8.4.1 Hyperglycaemia in elderly

Assessing the outcome variable of study III, hyperglycaemia, in the context of weight change, literature is limited. It has however been shown that subjects who are older, overweight, and have other diabetes risk factors are more likely to progress from hyperglycaemia to diabetes (174). This is well in hand with the findings from Alexander et al. showing that fasting glucose, diabetes risk, and systolic blood pressure values show a linear relationship with ageing and the BMI (175).

Focusing specifically on hyperglycaemia, however not in the context of weight change as such, but merely in relation to weight, the concept of being metabolically obese, despite a normal weight, has been proposed to explain the high risk of hyperglycaemia and T2DM in some normalweight subjects (176). This is confirmed by Meigs et al. in 2006 concluding how the incidence of T2DM is higher among individuals with normal weight but with insulin resistance or the metabolic syndrome than in overweight individuals who do not have insulin resistance or the metabolic syndrome (160,161). Thus the relevance of assessing risk in all weight groups are of importance.

In study III we only assess hyperglycaemic risk in the presence of IFG and HbA1c. However, in a meta-analysis of prospective studies published between 1979 and 2004, annualised incidence rates of progression to diabetes in patients with isolated IGT (4-6%) or isolated IFG (6-9%) were lower than those with both IFG and IGT (15-19%) (79). indicating that although both IFG and IGT are insulin-resistant states, they differ in their site of insulin resistance (76,177). These observations are confirmed in a Swedish longitudinal study on post-menopausal women concluding that IGT and IFG are two diagnostic entities which overlap only partly. They find a larger fraction of women with IGT than IFG and conclude

that choosing to measure only FPG would result in missing a pre-diabetic state (81). It could therefore be argued, that we in study III only partly cover the fraction of elder women at pre-diabetic risk by only assessing FPG.

8.5 Limitations in Longitudinal Research

Each manuscript has its respective limitations, which are discussed in the individual papers. This section is thought as a description of the overall pitfalls associated with the use of epidemiological data.

The PERF cohort is a homogeneous group of elderly Danish women; same age-group, gender, same rights to health care services (government funded health care) with all contact to the health care system linked to a unique social security number. The work presented in this thesis is built largely upon the use of register-based information of outcomes which are obtained from Danish national registries applying this unique construct. The national registries can be used to obtain figures for the most serious part of morbidity leading to hospitalization or death, but an overall assessment of disease incidence can only be achieved by examination of representative samples of the population as done in the PERF cohort. A clear advantage of applying register based data is the very limited loss to follow-up and also the low information bias, as it is not a self-reported diagnosis but a medically confirmed diagnosis applied in as the end-point in the statistical analysis. However, one limitation needs to be considered, when applying data specifically from the National Diabetes Registry. As reported on a global level, diabetes is greatly underdiagnosed. This is also the case in Denmark. Findings from the Inter99 study indicated that more than half of the persons with diabetes according to the diagnostic criteria are undiagnosed (178). So, the register is not complete in the clinical sense that all diabetics are actually diagnosed. The misclassification of this discrete variable will lead to misclassification, which will alter the calculated risk estimates. Green et al. validated the register in 2014 and found that approximately 20% of the registrations in the registry may be false positive inclusions of persons with frequent measurements of blood glucose without having diabetes. They, therefore, recommended changes for improving validity, by reducing the impact of current sources of bias and misclassifications (179). The diabetes register has later been terminated and will re-open in another format.

One last note to be touched upon in relation to metabolic risk factors; confounding. Rothman describes confounding in its most simple term as a confusion of effects (4). In

relation to the presented studies, a confusion of effect, or 'what is the actual exposure' are relevant to touch upon as obesity and insulin resistance are so interlinked. In study IV of this thesis, the reason for weight loss is not known and the associations between obesity and hyperglycaemia are constructed in a way, where obesity is assumed to precede hyperglycaemia. However, a possible risk of misinterpreting weight relations as a result of hyperglycaemia risk (or the other way around) is possible, as the follow-up period is 13 years. An obese woman with normoglycaemic levels at baseline developing hyperglycaemia over time (prior to follow-up), might be advised to lose weight as a result of the hyperglycaemic levels, thereby initiating a weight-loss based on the outcome parameter of the study. She will most likely end up in 'obese weight losing' category thereby increasing the risk estimate in the weight losing category, as she has actively lost weight as a result of the outcome parameter. This is considered to greatly confound the risk estimate attained in this type of study setup.

9 CONCLUDING REMARKS

This thesis contributes to enhance the information currently available from longitudinal research studies in elder women.

The PERF cohort profile demonstrates that the PERF cohort is representable for the elderly Danish female population and that the characteristics of the PERF II follow-up participants did not vary significantly from women from the PERF I cohort, who were not followed up.

The studies presented in the thesis specifically showed that:

- Elderly women defined with MetS showed an increased the risk of future T2DM and CVD. This risk increased when the reference group only comprised women holding no risk factors for MetS.
- Women not defined with MetS, however still holding metabolic risk factors, which are used to define the syndrome, were also at increased risk of T2DM or CVD compared to women with no MetS risk factors.
- Overweight and obese elderly women had a significantly increased risk of hyperglycaemia compared to normalweight elderly women.
- Losing weight in late life had a positive effect on the risk of hyperglycaemia in overweight and obese women, while further weight gain increased the risk of hyperglycaemia.
- Increased MMP-mediated tissue degradation was identified as an independent risk factor of increased all-cause mortality measurable up to nine years prior to death.
- MMP-mediated tissue degradation may be an important predisposition for the cause of disease and subsequent mortality.

10 FUTURE PERSPECTIVES

The work presented in this dissertation only touches upon a fraction of the pressing research questions related to upholding a healthy ageing process. One major risk indicator when discussing MetS, T2DM and CVD is clearly missing from the presented analysis, namely insulin. Insulin, was not measured as part of the biomarker panel available from the PERF dataset, however, new funding has made it possible to measure insulin within the full cohort both at baseline (PERF I) and follow-up (PERF II). This will give us valuable insights into associations between insulin and weight in elderly women. It will further help in the investigation of how insulin levels, and insulin resistance collate with weight, waist circumference and BMI, but also hand-grip strength; all parameters which have been touched upon in this thesis. Their associations with insulin would contribute to a more thorough evaluation of sarcopenic obesity and also help us to determine if obesity or insulin resistance, per se, is the actual driver of the metabolic disorders associated with the MetS preceding T2DM and CVD in elder women.

In relation to the further exploration of collagens, as a scaffold for disease, and their association with metabolic disorders, collagen type VI should be further explored. The C-terminal pro-peptide of collagen type VI has been shown to function as the hormone endotrophin, which is associated with the MetS. Endotrophin is a co-stimulator of pathological paths within the adipose tissue ultimately triggering fibrosis and inflammation leading to enhanced insulin resistance and metabolic dysfunction (180). It would be interesting to explore this association further, by analysing if increased endotrophin levels are a predecessor for T2DM by correlating delta-HOMA-IR values and endotrophin levels applying samples from PERF I and PERF II.

Moreover, an interesting parameter could be to address biomarkers reflecting protein formation, as the ratio between formation and degradation of the same protein may also add additional knowledge to what is currently known regarding ECM remodelling in ageing. The neo-epitope biomarker could also be combined with other existing or novel biomarkers of inflammation and insulin resistance for improving accuracy.

As a final remark on the future employment of the PERF cohort, the potential that this cohort has to explore the use of personalized healthcare are promising. The term personalized healthcare is increasingly used and has been extensively elaborated upon as

the worldwide population is ageing. Personalized healthcare is thought to limit costs of treatment by only treating the group of individuals actually responding to the medication available. With this approach in mind, prediction of response to treatment and further demonstration of efficacy of a drug candidate are among the most difficult challenges in drug development. Based on the current data, C1M seems to have the prognostic potential for prediction of mortality, and may therefore possibly help identifying patients with increased tissue degradation, which could possibly benefit from treatment.

11 EPILOGUE

In industrialised countries, health care systems are criticised, latest by the WHO, for being better designed to treat and cure acute conditions rather than to manage and minimize the consequences of the chronic states of disease, which are most commonly prevalent in older people (11,181–183). This paradox is one of the main reasons why the health of older people is not keeping up with the increasing longevity (184). Reducing severe disability from disease is a key feature of controlling the economic burden associated with the demographic changes. Health systems need better data to understand the health risks faced by older people and to target appropriate prevention and intervention strategies. Knowledge of risk factors is pivotal in this strategy (11).

The vision for following up the PERF cohort was to specifically aid in these strategies by combining longitudinal data analysis, register-based information, and panels of well-described biomarkers. But also to apply novel neo-epitope biomarkers, such as C1M, which help us to provide a broader definition of what underlines a chronic state of excessive tissue remodelling. Combined, all of this information will aid in gaining valuable information needed in order to obtain a further compression of morbidity of the ageing population, and thereby increase the quality of life at older age.

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