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Subclinical inflammation and type 2 diabetes mellitus in longitudinal cohort studies

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“Der Zweifel ist der Beginn der Wissenschaft. Wer nichts anzweifelt, prüft nichts. Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind“

Teilhard de Chardin

Zusammenfassung

Die Ursachen für die Entwicklung des Typ 2 Diabetes liegen in der Kombination aus Insulinresistenz, einer unzureichenden Kompensation durch Insulinsekretion und einer schließlich progredient auftretenden β-Zell-Fehlfunktion. Das Risiko, an Typ 2 Diabetes zu erkranken, wird durch verschiedene Faktoren beeinflusst, wobei Adipositas einer der wichtigsten Faktoren darstellt. Dabei wird das angeborene Immunsystem aktiviert, wobei es zu einer chronischen und geringgradigen Erhöhung der Konzentration zirkulierender Immunmediatoren kommt. Es ist bekannt, dass die erhöhte Konzentration von pro-inflammatorischen Immunmediatoren mit einem steigenden Risiko an Typ 2 Diabetes zu erkranken assoziiert ist. Die Rolle von anti-inflammatorischen Immunmediatoren in der Entwicklung des Typ 2 Diabetes ist allerdings bisher unklar. Deshalb beschäftigt sich diese Dissertation mit der Analyse der Serumkonzentrationen von vier verschiedenen anti-inflammatorischen Immunmediatoren im Zusammenhang mit Typ 2 Diabetes: Interleukin-1 Rezeptorantagonist (IL-1Ra), Adiponektin, Macrophage-inhibitory cytokine-1 (MIC-1) und Secreted frizzled-related Protein 5 (Sfrp5).

Unsere Daten aus der Whitehall II Kohorte zeigen, dass 13 Jahre vor der Manifestation des Typ 2 Diabetes die Konzentration von IL-1Ra erhöht und die von Adiponektin im humanen Serum erniedrigt war. Sechs Jahre vor der Diagnose des Typ 2 Diabetes stieg die IL-1Ra-Konzentration steil an, während die Adiponektin-Konzentration während des gesamten Beobachtungszeitraums gleichmäßig abfiel. Der Abfall in der Adiponektin-Konzentration vor der Diagnose des Typ 2 Diabetes war bei Frauen stärker ausgeprägt als bei Männern sowie stärker bei Patienten mit Typ 2 Diabetes, die bei der Diagnose ein geringeres Alter aufwiesen im Vergleich zu solchen mit einem höheren Alter. Adipositas hatte nur einen geringen Einfluss auf die Konzentration von IL-1Ra, aber eine erhebliche Auswirkung auf die von Adiponektin. Die Konzentration beider Immunmediatoren war bei Frauen höher als bei Männern. Dies deutet darauf hin, dass Geschlechtshormone einen Einfluss auf deren Freisetzung haben könnten. Wir vermuten, dass eine Hochregulation des pro-inflammatorischen Immunmediators Interleukin-1β (IL-1β), die sowohl durch Lebensstilfaktoren als auch genetische Prädisposition induziert werden kann, die beobachteten Veränderungen in den Konzentrationen von IL-1Ra und Adiponektin verursacht haben könnte. Wir

nehmen an, dass der Anstieg in der Konzentration des IL-1β-Antagonisten IL-1Ra ein Indikator erhöhter IL-1β-Konzentration und somit ein vergeblicher Versuch des Immunsystems sein könnte, den schädlichen metabolischen und inflammatorischen Effekten dieses Immunmediators entgegenzuwirken. Der Abfall der Konzentration des insulin-sensibilisierenden Adiponektin hingegen scheint die Insulinresistenz weiter zu begünstigen. Es kommt zu steigenden Glukosespiegeln, die wiederum zu einer Hochregulation von IL-1β führen. Von daher scheint dieses Zusammenspiel von metabolischen und immunologischen Faktoren einen Teufelskreis voranzutreiben, der letztendlich in der Entwicklung des Typ 2 Diabetes endet.

In weiteren Untersuchungen konnten wir zeigen, dass in Personen vor der Entwicklung des Typ 2 Diabetes die Konzentration von MIC-1 erhöht und nicht wie vermutet erniedrigt vorlag. Allerdings war die Assoziation zwischen erhöhter MIC-1-Konzentration und dem höheren Risiko, an Typ 2 Diabetes zu erkranken, von verschiedenen anderen Risikofaktoren für Typ 2 Diabetes abhängig. Dies deutet darauf hin, dass MIC-1 nicht in ähnlicher Weise reguliert wird wie Adiponektin.

Ferner legen unsere Daten dar, dass Sfrp5 positiv mit Insulinresistenz (gemessen als HOMA-IR) und oxidativem Stress assoziiert war und daher dessen Konzentration im humanen Blut offenbar anders reguliert wird als Adiponektin. Im Vergleich zu früheren Beobachtungen im Fettgewebe der Maus, könnte dieser unerwartete Befund allerdings auch auf spezies- und gewebespezifische Unterschiede in der Regulation von Sfrp5 zurückzuführen sein.

Diese Dissertation trägt zum Verständnis der komplexen Interaktion zwischen anti-inflammatorischen Immunmediatoren und metabolischen und immunologischen Störungen vor der Manifestation des Typ 2 Diabetes bei. Aufbauend auf unseren Befunden sind jedoch weitere Analysen des zeitlichen Ablaufs anderer pro- und anti-inflammatorischer Immunmediatoren vor der Diagnose des Typ 2 Diabetes notwendig, um die Mechanismen, die zur Entwicklung des Typ 2 Diabetes beitragen, weiter im Detail zu charakterisieren. Für diese Analyse wären der pro-inflammatorische Immunmediator IL-1β – als postulierter Auslöser dieses Teufelskreises – und der anti-inflammatorische und vor kardiovaskulären Krankheiten schützende Immunmediator MIC-1 – als möglicher diabetesprotektiver Immunmediator – interessante Kandidaten.

Summary

Type 2 diabetes is caused by a combination of insulin resistance, an inadequate compensatory insulin secretion and eventually progressive β-cell failure. The risk of developing type 2 diabetes is influenced by several risk factors such as obesity that are able to activate the innate immunity resulting in a chronic and slight increase of circulating levels of immune mediators. It is well-known that increased levels of pro-inflammatory immune mediators are associated with an increased risk of type 2 diabetes. However, the role of anti-inflammatory immune mediators in the development of type 2 diabetes is still unclear. Therefore, this doctoral thesis focuses on human serum levels of four anti-inflammatory immune mediators in the context of type 2 diabetes: interleukin-1 receptor antagonist (IL-1Ra), adiponectin, macrophage inhibitory cytokine-1 (MIC-1) and secreted frizzled-related protein 5 (Sfrp5).

Our data from the Whitehall II cohort demonstrate that IL-1Ra levels were increased and adiponectin levels were decreased 13 years before the manifestation of type 2 diabetes. IL-1Ra levels steeply increased six years before the diagnosis of type 2 diabetes, whereas adiponectin levels followed a linear decline during the whole observation time. However, decreases in adiponectin levels before the diagnosis of type 2 diabetes were more pronounced for women than for men and were also stronger in patients with type 2 diabetes with younger compared to older age at diagnosis. Obesity had only little impact on IL-1Ra levels but substantially influenced the concentrations of adiponectin. Levels of both immune mediators were higher in women than in men indicating that sex hormones may have an impact on their release. We hypothesise that an upregulation of the pro-inflammatory immune mediator interleukin-1β (IL-1β) which can be induced by both lifestyle-based risk factors for type 2 diabetes and genetic predisposition may have caused the observed changes in IL-1Ra and adiponectin levels. We assume that an increase of the IL-1β antagonist IL-1Ra might be an indicator of elevated IL-1β levels and represents a futile attempt by the immune system to counterregulate deleterious metabolic and inflammatory effects of this immune mediator. Decreases of the insulin-sensitising adiponectin might further promote insulin resistance. As a consequence, increased levels of glucose in turn lead to a further upregulation of IL-1β. Thus, the interplay between immunological

and metabolic factors may fuel a vicious circle that finally results in type 2 diabetes.

Further analyses reveal that serum levels of MIC-1 were increased, not decreased, in individuals before type 2 diabetes but its association with higher risk of type 2 diabetes depended on several risk factors for type 2 diabetes. This indicates that MIC-1 is not regulated in a similar way as adiponectin.

In addition, we found that Sfrp5 was positively correlated with insulin resistance (assessed as HOMA-IR) and oxidative stress and therefore appears to be differentially regulated in human blood than adiponectin. Compared to previous observations in adipose tissue of mice, this unexpected finding might be explained by species- and tissue-specific differences in the regulation of Sfrp5.

This doctoral thesis contributes to the understanding of the complex interaction between anti-inflammatory immune mediators and metabolic and immunological disturbances before the manifestation of type 2 diabetes. However, further investigations of the time-course of other pro- and anti-inflammatory immune mediators before the diagosis of type 2 diabetes are necessary to characterise the mechanisms contributing to the development of type 2 diabetes in more detail. The most interesting candidates for these analyses are the pro-inflammatory immune mediator IL-1β – as postulated trigger of the vicious circle - and the anti-inflammatory and cardioprotective immune mediator MIC-1 - as possible diabetes-protective immune mediator.

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List of abbreviations

Acrp30	Adipocyte complement-related protein of 30-kDa
ADA	American Diabetes Association
ADIPOQ	Adiponectin gene
AP-1	Activator protein-1
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
CI	Confidence interval
CoA	Coenzym A
CRP	C-reactive protein
CVD	Cardiovascular disease
C1q	Complement factor
C2C12	Mouse myoblast cell line
C57BL/6J	JAX® mice strain (Jackson laboratories)
DCCT	Diabetes Control and Complications Trial
DPP	Diabetes Prevention Program
DPS	Diabetes Prevention Study
EASD	European Association for the Study of Diabetes
e.g.	Exempli gratia (example given)
ELISA	Enzyme linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Endoplasmic reticulum
Fig.	Figure
FoxO1	Fork-head box O1
FPG	Fasting plasma glucose
g	Gramme
GDF-15	Growth and differentiation factor-15
GLUT2	Glucosetransporter 2

HbA1c / A1c	Glycated haemoglobin A1c
HOMA-IR	Homeostatic model assessment - insulin resistance
HR	Hazard ratio
IAPP	Islet amyloid polypeptide
ic	Intracellular
IDF	International Diabetes Federation
IDPP	Indian Diabetes Prevention Program
i.e.	Id est
IFG	Impaired fasting glucose
IFN	Interferon
IGT	Impaired glucose tolerance
IKK	Inhibitor of κ kinase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IP-10/CXCL10	IFNγ-inducible protein 10 / C-X-C motif ligand 10
JNK	C-jun N-terminal kinase
kDa	Kilodalton
kg/m^2	Kilogramme per square metre
KORA	Cooperative health research in the Region of Augsburg
LPS	Lipopolysaccharide
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory macrophages
MCP-1/CCL2	Monocyte chemotactic protein-1/C-C motif ligand 2
mg/dl	Milligramme per decilitre
MIC-1	Macrophage inhibitory cytokine-1
MIF	Macrophage migration inhibitory factor
ml	Millilitre
mmol/l	Millimole per litre
MODY	Maturity-onset diabetes of the young
MONICA	Monitoring of trends and determinants in cardiovascular disease
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

ng/ml	Nanogramme per millilitre
NGSP	National Glycohemoglobin Standardisation Program
NGT	Normal glucose tolerance
NIK	NF-κB inducing kinase
Nlrp3	Nod-like receptor family, pyrin domain containing 3
OGTT	Oral glucose tolerance test
OR	Odds ratio
p	p-value
PDX-1	Pancreatic duodenal homoeobox 1
pg/ml	Picogramme per millilitre
PKC	Protein kinase C
PKR	Protein kinase R
RANTES/CCL5	Regulated on activation, normal T-cell expressed and secreted/C-C motif ligand 5
ROS	Reactive oxygen species
s	Secreted
SD	Standard deviation
Sfrp5	Secreted frizzled-related protein 5
SHBG	Sex hormone-binding globulin
TGF	Transforming growth factor
TNF	Tumour necrosis factor
µg/ml	Mikrogramme per millilitre
UPR	Unfolded protein response
U.S.	United States
vs.	Versus
WHO	World Health Organization
WHS	Women´s Health Study
Wnt5a	Wingless-type MMTV integration site family, member 5a
WOSCOPS	West of Scotland Coronary Prevention Study
2-h	2-hour
3T3-L1	Mouse embryonic fibroblast - adipose like cell line

Chapter 1

General Introduction

1.1 Epidemiology of type 2 diabetes mellitus

1.1.1 Definition of diabetes

Diabetes mellitus is a general term for several metabolic diseases that are all characterised by hyperglycaemia. The causes of hyperglycaemia are abnormalities in insulin secretion and/or insulin action. The definition of the different types of diabetes mellitus is based on their aetiopathogenesis. Diabetes mellitus is classified into subtypes:

(1) type 1 diabetes which can be attributed to immune-mediated β-cell destruction usually leading to reduced and ultimately absent insulin secretion and accounts for 5-10% diabetes cases;
(2) type 2 diabetes which is caused by a combination of resistance to insulin action and an inadequate compensatory response of insulin secretion and accounts for up to 90% of the diabetes cases;
(3) various other forms ranging from genetic defects of β-cell function such as MODY (maturity-onset diabetes of the young) or of insulin action to diseases of the exocrine pancreas and drug- or chemical-induced diabetes;
(4) gestational diabetes mellitus (1,2).

The treatment of diabetes depends on severity and type of diabetes. Some patients with diabetes can control their blood glucose levels by weight reduction, exercise and/or oral glucose- and lipid-lowering agents, while others need exogenous insulin supply for adequate glycaemic control (1).

Long-term macro- and microvascular complications of diabetes caused by chronic hyperglycaemia and other factors/mechanisms such as abnormal lipid metabolism or subclinical inflammation are the major problems of diabetes because they have a substantial impact on the quality of life and may lead to increased mortality. These complications include cardiovascular diseases (CVDs), retinopathy, nephropathy and neuropathy that can result in myocardial infarction, stroke, atherosclerosis, retinal damage with potential loss of vision, renal failure, foot ulcers and amputations (1,3).

1.1.2 Diagnosis of diabetes

Normal glucose tolerance (NGT) is defined by fasting plasma glucose (FPG) levels < 100 mg/dl and 2-hour (2-h) glucose levels in the oral glucose tolerance

test (OGTT) < 140 mg/dl. Currently, there are four criteria for the diagnosis of diabetes: (1) Glycated haemoglobin A1c (HbA1c or A1c) ≥ 6.5% (measured in a laboratory using a method that is National Glycohemoglobin Standardisation Program (NGSP) certified and standardised to the Diabetes Control and Complications Trial (DCCT) assay), (2) levels of fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/l) (fasting means no caloric intake for at least 8 hours), (3) levels of 2-hour glucose ≥ 200 mg/dl (11.1 mmol/l) during an OGTT (according to the recommendations of the World Health Organization (WHO), the glucose load contains the equivalent of 75 g anhydrous glucose dissolved in water), (4) a random concentration of plasma glucose ≥ 200 mg/dl (11.1 mmol/l) in combination with classic symptoms of hyperglycaemia or hyperglycaemia crisis. If no unequivocal hyperglycaemia is present, results from (1), (2) and (3) should be confirmed by repeat testing. The values for glucose are valid in venous plasma. Point-of-care testing is not sufficiently precise for diagnostic purposes (1,2,4,5).

Until 2009, the diagnosis of diabetes was based on FPG, 2-h plasma glucose in the 75 g-OGTT or random plasma glucose in combination with symptoms of diabetes. In 2009, the International Expert Committee including representatives of the American Diabetes Association (ADA), the International Diabetes Federation (IDF), and the European Association for the Study of Diabetes (EASD) recommended the use of the HbA1c test to diagnose diabetes with a cut-off point of ≥ 6.5% (6). The ADA adopted this criterion in 2010 (1) and the WHO also published this recommendation in 2011 (7).

The guideline of the German Diabetes Association recommends the measurement of glucose according to conventional glucose criteria for patients with diabetes with an HbA1c between 5.7 and 6.4%. An HbA1c ≥ 6.5% is sufficient to diagnose diabetes and an HbA1c below 5.7% indicates that the person is very unlikely to have diabetes. The measurement based on HbA1c may be insufficiently accurate for example in patients with chemical modifications of haemoglobin, inhibition of the glycation reaction or pregnancy and changed turnover of red blood cells (e.g., certain forms of anaemia) (4).

1.1.3 Prediabetes

Some individuals have blood glucose levels that do not meet the diabetes criteria, but excede the established thresholds of normal glucose levels. This

“intermediate” group of individuals is referred to as having prediabetes. FPG levels between 100 and 125 mg/dl (5.6-6.9 mmol/l) indicate impaired fasting glucose (IFG). The WHO defines a cut-off for IFG at 110 mg/dl (6.1 mmol/l) (2). A person is defined as having impaired glucose tolerance (IGT), if this person has a 2-h plasma glucose in the 75 g-OGTT between 140 and 199 mg/dl (7.8-11.0 mmol/l). Individuals with an HbA1c of 5.7 to 6.4% may be also referred to as having prediabetes. Individuals with IFG and/or IGT are at increased risk to develop diabetes and/or CVD (1,2,4).

1.1.4 Prevalence of type 2 diabetes

In a current estimate of the prevalence of diabetes worldwide, the number of people living with diabetes will rise from 366.2 million in 2011 to 551.8 million by 2030 (8). Possible causes for this increase in numbers of diabetes cases worldwide by 2030 are population growth, ageing of populations and urbanisation that are associated with lifestyle changes which result in obesity (9-11). Interestingly, 80% of people with diabetes live in low- and middle-income countries of whom 172 million people live in urban areas and 119 million people in rural areas. It is expected that this difference will be more pronounced in 2030 with 314 million people living in urban areas and 143 million in rural areas (8).

In its current atlas, the IDF reports a prevalence of 8% of diabetes cases among subjects aged between 20 and 79 years in Germany (8). The KORA (Cooperative health research in the region of Augsburg) S4 (1999-2001) and F4 surveys (2006-2008) are the first population-based studies in Germany using the OGTT to examine the prevalence of diabetes and prediabetes. The studies showed a high prevalence of impaired glucose homeostasis in younger and middle-aged German women and men (35-59 years). Both, the prevalence for previously and newly diagnosed diabetes was about 2%. Therefore, the proportion of previously unknown, newly diagnosed diabetes was 50% among all cases with diabetes (12). In the elderly German population (55-74 years), it is estimated that there are 270,000 new diagnosed type 2 diabetes cases each year in Germany (13). About 40% of this elderly population had disturbed glucose tolerance or diabetes in the KORA S4 study, whereby half of the total diabetes cases were undiagnosed again (14). The data on the prevalence of diabetes cases in Germans from the IDF should be regarded with caution because of the

heterogenous study design of the regional prevalence estimates that were used to calculate the total German diabetes prevalence in Germany. In addition, the proportion of unknown diabetes cases are often estimated (15). Similar problems hold true for worldwide estimations of the diabetes prevalence.

1.1.5 Risk factors for type 2 diabetes

The risk for developing type 2 diabetes is influenced by several factors as illustrated in Figure 1.

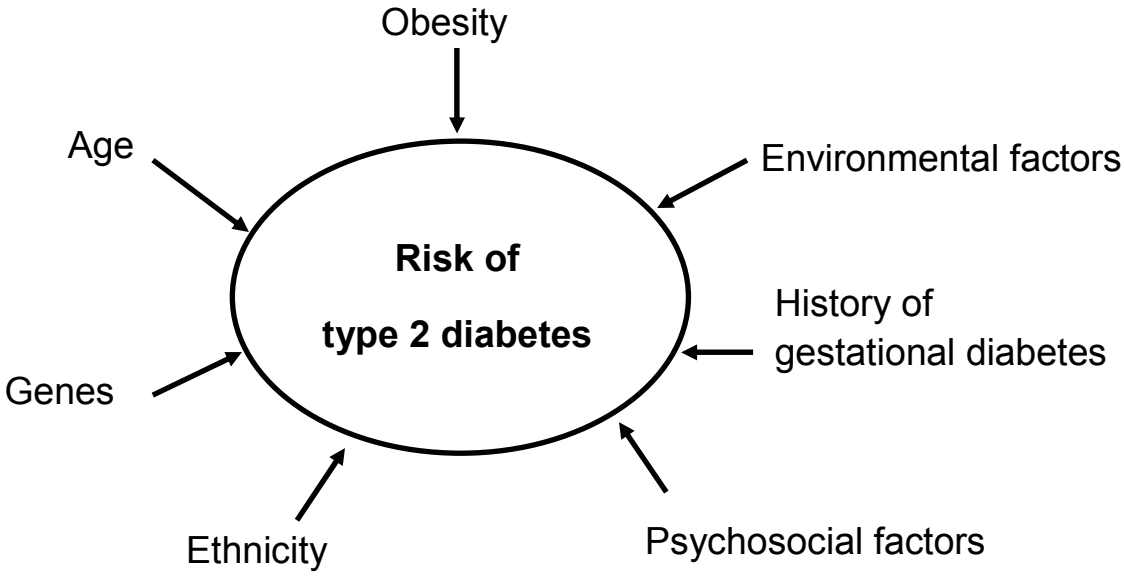

Figure 1: Factors increasing the risk of type 2 diabetes.

Obesity

One of the most important risk factors for type 2 diabetes is obesity. Obesity is defined as a body mass index (BMI) > 30 kg/m^2. The cause of obesity is an energy imbalance between calories consumed and calories expended. Worldwide, more than one in ten of the adult population is obese (16). Obesity is frequently associated with insulin resistance that is defined as a physiological condition characterised by a less effective insulin action to lower glucose levels. Therefore, weight loss is an important therapeutic objective for patients with diabetes or with high risk of type 2 diabetes (17). Diet and/or exercise are important components in the management of type 2 diabetes and prevention of type 2 diabetes in individuals at high risk (18). The Diabetes Prevention Study (DPS) in Finland, the

Da Qing IGT and Diabetes Study in China, the Diabetes Prevention Program (DPP) in the U.S. and the Indian Diabetes Prevention Program (IDPP) demonstrated that a low-caloric and low-fat diet and exercise for at least 30 minutes per day significantly reduced the risk of diabetes by 28.5 to 58.0% (19-22). In patients with type 2 diabetes with poor glycaemic control and a BMI greater than 35 kg/m^2, bariatric surgery may be considered. Bariatric surgery frequently leads to a remission of type 2 diabetes (23).

Age

People above 45 years of age are at an increased risk of type 2 diabetes (24). Moreover, different estimates and calculations of the prevalence of type 2 diabetes worldwide show that the number of adults with diabetes in developed and developing countries is higher in elderly compared to the younger subjects (8,9,12,13). There are many possible reasons for the increased incidence of type 2 diabetes that is observed with higher age. Reduced insulin sensitivity, decreased β-cell function and reduced proliferative capacity of β-cells may contribute to age-related glucose intolerance (25,26). However, recent studies indicated that biological aging per se may not be the reason for decreased insulin sensitivity, but rather reduced physical activity, increased fat mass and impaired mitochondrial function (27).

Genes

Individuals with a family history of type 2 diabetes are at an increased risk of type 2 diabetes. If a person has one first-degree relative with type 2 diabetes, the risk of diabetes is 14.3% for this subject compared to 3.2% for those without such a relative (28). However, only 10% of the genetic predisposition can be explained yet, although more than 40 genes that are associated with type 2 diabetes have been identified (29).

Ethnicity

Certain ethnic groups (e.g., Non-Hispanic Blacks, Hispanic/Latin Americans, Asian Americans and Pacific Islanders, and American Indians and Alaska Natives) have a higher risk of type 2 diabetes than European Whites. This difference in diabetes risk may be due to differences in genetic predisposition, but most studies

that investigated genetic susceptibility loci of type 2 diabetes were performed in populations of European origin, so that a direct comparison between ethnic groups is difficult (29).

Other factors

Psychosocial stress can also be a trigger factor for several diseases including diabetes. Individuals with psychosocial work stress or depressive disorders have an increased risk of type 2 diabetes (30-33). Traffic-related air pollution is associated with a higher risk of type 2 diabetes in German women (34). Many women who had gestational diabetes develop type 2 diabetes years later. The offspring are also at an greater risk for developing diabetes in later life. (35)

1.1.6 Pathogenesis of type 2 diabetes

The pathogenesis of type 2 diabetes is characterised by insulin resistance, the attempt to compensate for this insulin resistance by higher insulin secretion and eventually a progressive β-cell failure (36). The role of insulin resistance and β-cell dysfunction in the development of type 2 diabetes has been evaluated in several studies. In a prospective cohort study within the Whitehall II study including over 6,500 subjects without diabetes at baseline, 505 incident type 2 diabetes cases were diagnosed during a median follow-up of 8.2 years. The latter had reduced insulin sensitivity 13 years before type 2 diabetes diagnosis and showed a steep decrease in insulin sensitivity during the last five years before type 2 diabetes diagnosis compared to those who remained diabetes-free. Beta-cell function increased three to four years before type 2 diabetes diagnosis and then decreased until diagnosis (37). Moreover, in a seven-year prospective study of 714 initially non-diabetic Mexican-Americans, increased insulin resistance and decreased insulin secretion, both measured at baseline, were independent risk factors for type 2 diabetes in the 99 subjects who developed type 2 diabetes (38).

The imbalance between insulin secretion and insulin action is mainly triggered by a chronic positive energy balance due to high-caloric diet and lack of physical activity. The caloric overload leads to increased levels of blood glucose (“glucotoxicity”), fatty acids and/or triglycerides (“lipotoxicity”) (39) and immune mediators (“subclinical inflammation”) (40). In addition, other mechanisms including islet amyloids may also contribute to this imbalance. In the following

sections, glucotoxicity, lipotoxicity and islet amyloids are described in more detail. The role of subclinical inflammation in the development of type 2 diabetes is illustrated in section 1.2 of the “General introduction”.

Glucotoxicity

Typically, glucose is rapidly taken up by the pancreatic β-cell via the glucose transporter 2 (GLUT2). The glycolysis in the cytoplasm and the Krebs cycle in the mitochondria generate adenosine triphosphate (ATP) that is necessary for the release of insulin and the cell membrane polarisation (41). The oxidative glucose metabolism in the β-cells always produces reactive oxygen species (ROS). Enzymes such as catalase, superoxide dismutase and glutathione peroxidase normally detoxify these ROS (42).

Under conditions of glucose oversupply, more ROS are generated in β-cells which cannot be detoxified completely. This results in damage of cellular components of the β-cells (42).

Moreover, high concentrations of glucose induce the production and release of the proinflammatory cytokine IL-1β in human islets (43). Increased levels of IL-1β in turn contribute to β-cell apoptosis and impair glucose-stimulated insulin secretion through activation of c-Jun N-terminal kinases (JNK) (44). Glucose also induces the production of other proinflammatory cytokines such as tumour necrosis factor-alpha (TNFα) and monocyte chemotactic protein-1/C-C motif ligand 2 (MCP-1/CCL2) in human monocytes (45). Various further molecular mechanisms have already been described to underlie glucose-induced β-cell dysfunction such as formation of advanced glycation end products (46), endoplasmatic reticulum stress (47), direct impairment of insulin gene transcription and proinsulin biosynthesis (48,49), and reduced binding activity of pancreatic duodenal homoeobox 1 (PDX-1), a critical regulator of insulin promoter activity (50).

In addition, glucose might induce β-cell apoptosis in human islets by upregulation of the Fas-receptor (51). High glucose levels may further promote the development of insulin resistance via several mechanisms: (i) activation of protein kinase C (PKC) isoforms leading to decreased glucose uptake and glycogen synthesis in both liver and skeletal muscle cells, (ii) upregulation of the hexosamine pathway resulting in the inhibition of glucose uptake in skeletal

muscle, (iii) altered glycosylation of proteins involved in glucose metabolism and (iv) malonyl-CoA dependent inhibition of lipid oxidation indicating that glucosetoxicity and lipotoxicity are linked to each other (“glucolipotoxicity”) (52).

Lipotoxicity

Increased levels of fatty acids are associated with a higher risk of type 2 diabetes in humans (53,54) The increased intake of fat in the diet enhances the storage of triglycerides in adipose tissue and ectopically in other tissues such as skeletal muscle, liver and pancreas (60).

Adipocytes which store a higher amount of triglycerides have a differential expression of pro- and anti-inflammatory immune mediators. This results in a shift towards higher levels of pro-inflammatory immune mediators and thus may contribute to a low-grade inflammation (55).

Cross-sectional studies showed that the triglyceride concentration within skeletal muscle cells (intramyocellular lipids) negatively correlates with insulin sensitivity in various populations of sedentary individuals (56-58). In contrast, physical activity increases both intramyocellular lipid content and insulin sensitivity (“training paradox”). Therefore, the intramyocellular lipid content can serve as energy source during intensive aerobic exercise but can also reflect an imbalance in energy intake and demand in sedentary insulin-resistant humans. In the latter, causes for the accumulation of intramyocellular lipids might be insufficient lipid oxidation and impaired mitochondrial function. Increased levels of intramyocellular lipids activate isoforms of PKC and nuclear factor ´kappa-light-chain-enhancer´ of activated B-cells (NF-κB) which inhibit insulin signal transduction resulting in decreased glucose uptake and phosphorylation (59,60).

The accumulation of lipids in liver cells (intrahepatocellular lipids) is called nonalcoholic fatty liver. Increased levels of intrahepatocellular lipids are negatively correlated with whole body and hepatic insulin sensitivity. Activated isoforms of PKC and NF-κB interfere with insulin signal transduction resulting in insulin resistance. In addition, lipogenesis is stimulated leading to increased levels of intrahepatocellular lipids and very low density lipoproteins. The dysregulation of the transcription factor Fork-head box O1 (FoxO1) is responsible for increased gluconeogenesis (60).

The amount of lipids in pancreatic islets correlate negatively with β-cell function and therefore pancreatic lipids might contribute to β-cell dysfunction (61,62)

Taken together, hyperglycaemia and hyperlipidaemia seem to be linked to each other. Glucolipotoxicity summarises the deleterious effects of hyperglycaemia and hyperlipidaemia that result in insulin resistance and impaired functions of muscle, liver and pancreatic β-cells (60).

Islet amyloids

Type 2 diabetes is characterised by an extracellular accumulation of amyloids in pancreatic islets (63). Islet amyloids consist of amyloid polypeptides (IAPP) that are co-secreted with insulin (64). IAPPs form amyloid deposits that lead to β-cell death and inhibit glucose-stimulated insulin secretion. Therefore, IAPP may be implicated in the pathogenesis of diabetes (65,66).

The aforementioned mechanisms are not independent, but can interact with each other. They can contribute to abnormal mitochondrial function and endoplasmic reticulum stress that were also found to be involved in the pathogenesis of type 2 diabetes.

Abnormal mitochondrial function

Mitochondria are the power stations within cells and their key function is the production of ATP (67). Type 2 diabetes can be associated with a reduced mitochondrial function in β-cells, skeletal muscle, liver and adipose tissue (68). It could be shown that a reduced mitochondrial function in β-cells can reduce insulin secretion in these cells (69). In skeletal cells, the levels of fatty acyl coenzym A (CoA) and diacylglycerol are increased by the impairment of mitochondrial fatty acid β-oxidation. These metabolites affect insulin signalling pathways and mediate insulin resistance (70). Similar to muscle cells, liver cells accumulate intracellular fatty acids by reduced mitochondrial fatty acid β-oxidation leading to decreased glycogen synthesis and increased gluconeogenesis (71). Mitochondrial dysfunction in brown adipose tissue (BAT) is associated with impaired thermogenesis and energy expenditure and contributes to insulin resistance (68,72).

Endoplasmic reticulum (ER) stress

The ER is responsible for protein folding and controls the quality of newly synthesised proteins (73). A balance between ER protein load and ER folding capacity is required to fold proteins in the correct way (74). ER stress is caused by the accumulation of misfolded proteins in the ER. Thereby, the unfolded protein response (UPR) is activated (75). In the development of type 2 diabetes, ER stress caused by metabolic overload mediates β-cell dysfunction and insulin resistance. The UPR becomes permanently activated when the demand for insulin overwhelms the folding capacity of the ER. Stimuli for the accumulation of misfolded proteins within the ER lumen of β-cells are fatty acids, glucose and IAPP (68,76).

1.2 Subclinical inflammation and type 2 diabetes

1.2.1 Definition of subclinical inflammation

Inflammation is classically characterised by five cardinal signs: redness, swelling, heat, pain and loss of function (77). This kind of inflammation represents a rapid response to injury or infection. After removing or neutralisation of the damaging agent and/or repair of the injured tissue, inflammation is resolved (78).

In comparison with classic inflammation, subclinical inflammation is of different nature while there is no generally accepted definition of subclinical inflammation. However, it is characterised by a chronic and low-grade increase of immune mediators. Subclinical inflammation appears gradually and remains unsolved over time (78). In Figure 2, a possible model of the development of subclinical inflammation in the context of obesity as main risk factor of type 2 diabetes is shown.

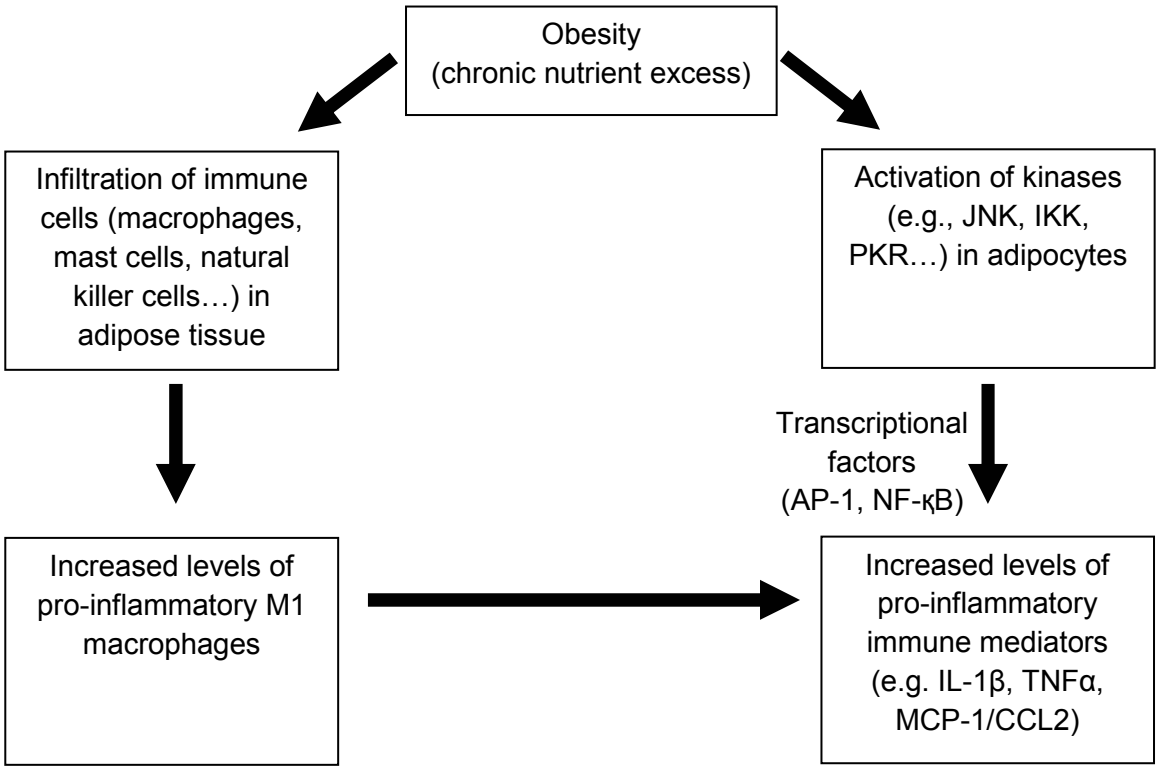

Figure 2: Model of the development of subclinical inflammation in obesity. AP-1: transcriptional factor activator protein-1, IKK: inhibitor of κ kinase; IL-1β: interleukin-1β, JNK: c-jun N-terminal kinase, MCP-1/CCL2: monocyte chemotactic protein-1/C-C motif ligand 2, NF-κB: nuclear factor ´kappa-light-chain-enhancer´ of activated B-cells, PKR: protein kinase R, TNFα: tumour necrosis factor-α.

A main trigger of subclinical inflammation is nutrient excess (obesity) leading to an activation of different kinases such as JNK, inhibitor of κ kinase (IKK) and protein kinase R (PKR) in adipocytes that in turn regulate the expression of different pro-inflammatory immune mediators such as IL-1β, TNFα and MCP-1/CCL2 through activation of transcriptional factors (transcriptional factor activator protein-1 (AP-1) and NF-κB (78). Moreover, there is an increased infiltration of immune cells into adipose tissue in obese individuals. Because of adipocyte death, macrophages might invade adipose tissue in order to remove dead cells and remodel the afflicted tissue regions. There are two types of macrophages: M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages whereby the amount of M1 macrophages is increased in obesity leading to an elevated expression of pro-inflammatory immune mediators. Multiple other types of immune cells such as mast cells and natural killer cells are also increased in response of obesity. In sum, subclinical inflammation is characterised by increased expression of pro-inflammatory immune mediators mediated by multiple signalling pathways and caused by nutrient excess (Figure 2) (78).

Apart from obesity as one main trigger of subclinical inflammation, there are several other risk factors of type 2 diabetes that have been found to activate innate immunity. These include physical inactivity, psychosocial stress and unfavourable diet whose association with the risk of type 2 diabetes (79) may be at least partially mediated by subclinical inflammation as described for obesity.

1.2.2 Immune mediators

Immune mediators comprise a diverse group of molecules with a wide range of functions in immune responses. Historically, they are subdivided in several classes such as acute-phase proteins, cytokines, chemokines and adipokines.

Acute-phase proteins comprise C-reactive protein (CRP), serum amyloid P component and serum amyloid A protein among others all of which are released from the liver after an acute-phase stimulus such as infection or trauma. Determination of CRP concentrations is most commonly used in clinical practice because the level of this dominant acute-phase protein increases immediately at onset of infection or tissue damage (80-82).

Cytokines are small cell-signalling molecules that can act in an autocrine, paracrine or endocrine manner. Cytokines control several cellular functions such as proliferation, phagocytosis, antigen presentation, antibody production, differentiation, regulation of cell death and interact with metabolic pathways. The major groups of cytokines are interleukins (IL), interferons (IFN) and tumour necrosis factor (TNF) (83). Many immune mediators belong to this class because they are secreted by immune cells. However, these immune mediators are often also secreted by other cells and/or have other functions such as IL-6 that is also secreted by muscle cells in response to muscle contraction (84).

Chemokines form a subgroup of cytokines which cause a directed migration of leukocytes along a chemokine concentration gradient. Chemokines can stimulate different cell types and enhance the release of several immune mediators. They are involved in different disorders such as allergy and atherosclerosis. Regarding the position of cysteine residues in the N-terminal region, chemokines are divided into four families: α chemokines (CXC), β chemokines (C-C), C-chemokines and CX3C chemokines (83,85,86).

Adipokines are immune mediators which are secreted mainly or exclusively by the adipose tissue such as adiponectin and leptin. Adipose tissue does not only store energy, but it also secretes a variety of factors into the local environment as well as into the circulation. The accumulation of macrophages into adipose tissue might be responsible for this enhanced secretion of most factors. Therefore, a complex communication between the adipose tissue and other organs is possible (87). The size of the adipocytes is directly associated with the amount of secreted immune mediators (55). Examples for immune mediators that are released by adipose tissue are IL-18, macrophage migration inhibitory factor (MIF), IFNγ-inducible protein 10/C-X-C motif ligand 10 (IP-10/CXCL10) and regulated on activation, normal T-cell expressed and secreted/C-C motif ligand 5 (RANTES/CCL5) (88-91).

1.2.3 Pro-inflammatory immune mediators

In the year 1998, the association between type 2 diabetes and increased circulating levels of pro-inflammatory immune mediators was described for the first time in an epidemiological study (92). Further reports extended this observation from cross-sectional to prospective studies and showed that a chronic activation of

the innate immune system leads to a subclinical, low-grade inflammation that is involved in the pathogenesis of type 2 diabetes (93-95).

The first immune mediator that was assessed to predict the development of type 2 diabetes was the acute-phase protein ***C-reactive protein (CRP)***. In the West Of Scotland COronary Prevention Study (WOSCOPS) the predictive value of CRP for the development of type 2 diabetes was assessed. For this investigation, 5,245 middle-aged men were included of whom 127 developed type 2 diabetes. In a univariate analysis, it could be shown that baseline CRP levels were an important predictor for the development of type 2 diabetes (hazard ratio [HR] for an increase of 1 standard deviation (SD) = 1.55; 95% confidence interval (CI) 1.32-1.82; $p<0.0001$). In a multivariate analysis, CRP remained a predictor of the development of type 2 diabetes independently of other risk factors such as baseline BMI, fasting triglyceride and glucose concentrations. Moreover, a stepwise increase in risk across CRP quintiles showed that in a multivariate analysis the highest quintile was associated with a 2.46-fold risk of developing type 2 diabetes (95% CI 1.20-5.04) in comparison with the lowest quintile. Therefore, in this study CRP predicted the development of type 2 diabetes in middle-aged men independently of other risk factors (96). The same could be shown for middle-aged women. In a prospective, nested case-control study within the Women´s Health Study (WHS) including 550 middle-aged women, elevated levels of CRP predicted the development of type 2 diabetes (97). However, a recent case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk cohort showed that CRP is not associated with type 2 diabetes after adjustment for central adiposity, markers of liver function and adiponectin. A meta-analysis of prospective studies on the association between CRP and type 2 diabetes gave evidence that there is a reverse causation. As the association between CRP and diabetes is strongly attenuated after adjustment for baseline hyperglycaemia it is possible that rising CRP levels might be associated with impairment in glucose control. Therefore, increased CRP levels might be a consequence rather than cause of hyperglycaemia (98).

The role of ***interleukin-6 (IL-6)*** in the development of type 2 diabetes is controversial (99,100). Some scientists believe that IL-6 does not have a beneficial effect on insulin sensitivity and glucose homeostasis (99), others claim the

opposite (100). IL-6 released from liver and adipocytes promotes insulin resistance (99,101,102). However, IL-6 released by skeletal muscle for example during exercise seems to improve insulin sensitivity (100). In a prospective, nested case-control study including 550 middle-aged women, baseline levels of IL-6 were higher in those who subsequently developed type 2 diabetes compared to those who remained diabetes-free ($p<0.001$). Women in the highest quartile of IL-6 had a 7.5-fold higher risk to develop type 2 diabetes (95% CI 3.7-15.4) compared to those in the lowest one. In a multivariate analysis, the relative risk for the highest vs. the lowest quartile was 2.3 (95% CI 0.9-5.6; p for trend = 0.07). After adjustment for fasting insulin level and limitation to women with an HbA1c equal or below 6.0%, similar results were observed (97).

Interleukin-1β (IL-1β) plays a central role in the development of type 2 diabetes. In murine and human adipocytes and rat hepatocytes, IL-1β is described to induce insulin resistance *in vitro* (103-105). In addition, IL-1β promotes β-cell apoptosis in pancreatic islets of rats *in vitro* (106). A nested case-cohort study within the EPIC-Potsdam study including over 27,000 individuals showed that increased levels of IL-6 and detectable levels of IL-1β were associated with greater risk to develop type 2 diabetes (odds ratio [OR] 3.5, 95% CI 1.1-11.2) (107).

Other pro-inflammatory immune mediators associated with type 2 diabetes are ***interleukin-18 (IL-18)*** and ***monocyte chemotactic protein-1/C-C motif ligand 2 (MCP-1/CCL2)***. In a prospective case-cohort study within the population-based MONItoring of trends and determinants in CArdiovascular disease (MONICA)/KORA study including 2,225 middle-aged women and men it could be shown that increased levels of IL-18 were associated with greater risk of type 2 diabetes after adjustment for several other risk factors such as age and BMI. Study participants in the highest quartile had a 1.73-fold higher risk to develop type 2 diabetes (95% CI 1.25-2.40) compared to those in the lowest one after adjustment for several risk factors such as age, BMI, CRP and IL-6. Interestingly, study participants with increased levels of both IL-18 and IL-6 or IL-18 and CRP, respectively, had the highest risk to develop type 2 diabetes (108). In the same prospective case-cohort study within the MONICA/KORA cohort increased levels of MCP-1/CCL2 contributed to the risk to develop type 2 diabetes. Study participants in the highest quartile of MCP-1/CCL2 had a 1.48-fold increased risk

to develop type 2 diabetes compared to those in the lowest one after adjustment for several risk factors such as age, BMI, CRP and IL-6 (p for trend = 0.026) (109).

Taken together, the role of pro-inflammatory cytokines in the development of type 2 diabetes is already well-characterised. The aforementioned five examples for pro-inflammatory immune mediators (CRP, IL-6, IL-1β, IL-18 and MCP-1/CCL2) demonstrate that increased levels of pro-inflammatory cytokines are associated with an increased risk of type 2 diabetes. However, there are wide gaps in our knowledge of the role of anti-inflammatory immune mediators in the development of type 2 diabetes.

1.2.4 Anti-inflammatory immune mediators

So far, adiponectin is the only anti-inflammatory immune mediator for which *increased* levels are associated with a *reduced* risk of type 2 diabetes. Adiponectin has insulin-sensitising properties and might be protective against type 2 diabetes (110).

The anti-inflammatory transforming growth factor-β1 (TGF-β1) has immunosuppressive effects (111) by inhibiting or reversing the activation of macrophages and by downregulating the production of pro-inflammatory cytokines, ROS and reactive nitrogen species (112,113). Consequently, *elevated* serum levels of TGF-β1 might associate with a *reduced* risk of type 2 diabetes. However, a prospective case-cohort study within the MONICA/KORA cohort showed that increased levels of TGF-β1 were associated with a *higher* and not *lower* risk of type 2 diabetes (age, sex and survey-adjusted HRs [95% CI] for increasing TGF-β1 tertiles: 1.0, 1.08 [0.83-1.42] and 1.41 [1.08-1.83]; p for trend = 0.012). After adjustment for other risk factors such as BMI, metabolic and lifestyle factors, the results were not altered significantly. Importantly, this study determined the levels of the latent form of TGF-β1 and could not measure the biologically active TGF-β1 which has a half-life of only two minutes (114).

Because of the difficulties to measure TGF-β1, the present data of this thesis focus on the time-course of two other anti-inflammatory immune mediators before the manifestation of type 2 diabetes. Adiponectin and interleukin-1 receptor antagonist (IL-1Ra) were previously found to be detectable in human serum by enzyme linked immunosorbent assay (ELISA) without any problems (115,116). The present data of this thesis also focus on the anti-inflammatory immune

mediators macrophage-inhibitory cytokine-1 (MIC-1) and secreted frizzled-related protein-5 (Sfrp5) in humans because of similarities in their regulation with adiponectin.

1.2.4.1 Interleukin-1 receptor antagonist (IL-1Ra)

The IL-1 cytokine family plays a key role in inflammation. One important member of the IL-1 family is IL-1Ra that is described as anti-inflammatory immune mediator. IL-1Ra competitively blocks the binding of the pro-inflammatory cytokines IL-1α and IL-1β to type I and type II IL-1 receptors without inducing any intracellular response (117,118). Multiple isoforms of IL-1Ra have been described: a secreted molecule from different immune cells (monocytes, macrophages, neutrophils, mast cells) (119-121), epithelial cells (122,123), skin keratinocytes (124), stromal cells (125-128), hepatocytes (129), adipocytes (130) and human β-cells (131) (s (secreted) IL-1Ra) and at least three intracellular molecules (ic (intracellular) IL-1Ra) that remain in the cytoplasma. The isoform sIL-1Ra competitively inhibits the local inflammatory effects of IL-1, whereas the function of icIL-1 isoforms within cells is less clear (132-134). There are several stimuli for the production of IL-1Ra such as lipopolysaccharides (LPS) (130), IL-1β and IFNβ (135). Interestingly, leptin reduces the levels of IL-1Ra, but increases the levels of IL-1β (131). A dysregulation in the critical balance of the pro-inflammatory IL-1β and the anti-inflammatory IL-1Ra seems to play an important role in the development of many diseases (136) such as type 2 diabetes (131).

As IL-1Ra has anti-inflammatory properties and antagonises the deleterious effects of IL-1β that contribute to the development of type 2 diabetes, the hypothesis arose that *increased* levels of IL-1Ra might be *protective* against type 2 diabetes. However, a nested case-control study within the Whitehall II cohort including 557 study participants showed that IL-1Ra levels are rather *higher*, but not *lower* in individuals who subsequently developed type 2 diabetes compared to those who remained diabetes-free (p=0.0006). Moreover, *elevated*, and not *decreased* levels of IL-1Ra are associated with an *increased* risk of type 2 diabetes (OR for 1-SD increase of IL-1Ra = 1.48 [95% CI 1.21-1.80]). This association was only attenuated after adjustment for 2-h plasma glucose (116). In contrast, the administration of IL-1Ra improved glucose tolerance as well as insulin secretion and prevented diabetes *in vivo* in C57BL/6J mice fed a high-

fat/high-sucrose diet. Islets from high-fat diet fed mice were protected from β-cell apoptosis and characterised by increased β-cell proliferation and improved glucose-stimulated insulin secretion after administration of IL-1Ra (137). A double-blind, parallel-group trial involving 70 patients with type 2 diabetes demonstrated that the administration of IL-1Ra improved glycaemia, β-cell secretory function and reduced markers of systemic inflammation (138).

Taken together, *increased* and not *decreased* levels of IL-1Ra are associated with a *higher* risk of type 2 diabetes. However, the administration of IL-1Ra seems to improve insulin sensitivity and β-cell function and to reduce subclinical inflammation.

1.2.4.2 Adiponectin

Adiponectin is a collagen-like protein that is also known as 30-kDa secretory protein Acrp30 (adipocyte complement-related protein of 30-kDa) as it is structurally similar to complement factor C1q. Adiponectin is exclusively expressed in adipocytes (139). There are several isoforms of adiponectin: three monomers of adiponectin form a trimer through association via their globular domain, four to six trimers can associate through their collagenous domains to form higher-order structures or oligomers that circulate at relatively high concentrations (μg/ml) in human blood. Adiponectin is present in the circulation as low- (trimers), medium- (hexamers) and high- (high-order multimers) molecular weight isoforms (140). Adiponectin plays a key role in the modulation of glucose and lipid metabolism in insulin-sensitive tissues in rodents, whereas its relevance in humans is less clear.

A range of epidemiological studies addressing the role of adiponectin for type 2 diabetes in humans have been performed. One study including 87 non-obese and 57 obese individuals showed that adiponectin levels were lower in obese subjects compared to non-obese subjects (141). In a study including Caucasians and Pima Indians, two groups with a high propensity for obesity and type 2 diabetes, adiponectin was inversely associated with body fat, fasting plasma insulin and 2-h glucose and directly associated with insulin sensitivity indicating that hypoadiponectinaemia contributes to insulin resistance and/or hyperinsulinaemia in these individuals (142). In a prospective, nested case-cohort study within the EPIC-Potsdam Study including over 27,000 individuals increased levels of adiponectin were independently associated with a reduced risk of type 2 diabetes

in apparently healthy subjects (OR for highest vs. lowest quartile = 0.3 [95% CI 0.2-0.7]) (143). In a prospective, nested case-cohort study within the Whitehall II cohort including 203 subjects higher levels of adiponectin were significantly associated with lower HbA1c levels independently of several risk factors ($p<0.05$) indicating that adiponectin is an independent predictor of diabetes and the degree of the impairment of glucose homeostasis (144).

The administration of adiponectin to mice decreased fasting glucose levels (145). In obese mice, the administration of adiponectin reduced insulin resistance in muscle cells and in the liver by increased β-oxidation and therefore led to reduced triglyceride content in these tissues (146,147). In addition, adiponectin raised glucose uptake in skeletal muscle cells after incubation of C2C12 myocytes with adiponectin *in vitro* (148). Moreover, adiponectin suppressed glucose production in isolated rat hepatocytes (145). Adiponectin also acts on the vascular system by reducing inflammation and may therefore mediate atheroprotective effects on the vasculature (149). It could be shown that adiponectin suppressed TNF-α-mediated inflammatory responses and CRP in human aortic endothelial cells in *vitro* (150,151). Thus, it has been postulated that adiponectin has antiatherogenic, anti-inflammatory and insulin-sensitising properties.

Taken together, *high* levels of adiponectin are associated with a *lower* risk of type 2 diabetes and adiponectin has several beneficial effects on metabolic and inflammatory processes. So far, adiponectin is the only anti-inflammatory immune mediator whose increased levels are associated with a decreased risk of type 2 diabetes.

1.2.4.3 Macrophage inhibitory cytokine-1 (MIC-1)

Macrophage inhibitory cytokine-1 (MIC-1) is also known as growth and differentiation factor-15 (GDF-15), placental transforming growth factor-β, prostate-derived factor and placental bone morphogenetic protein. MIC-1 is a member of the TGF-β superfamily and is secreted as a 28-kDa dimer after proteolytic cleavage of the dimeric pro-MIC-1 precursor in the ER (152,153). MIC-1 is primarily expressed in the placenta, prostate, kidney, liver and adipose tissue (154-156).

So far, there are no epidemiological studies on MIC-1 and its association with type 2 diabetes in humans. However, levels of MIC-1 can be increased in many

types of cancers in humans (breast, colorectal, prostate) (157). MIC-1 may have a potential role in the inhibition of macrophage activation because it has the ability to block LPS-induced TNF-α production by human macrophages *in vitro* (152). Macrophage numbers are increased in human type 2 diabetic islets (158) and the activities of macrophages accumulated in adipose tissue of obese individuals might contribute to the pathogenesis of obesity-induced insulin resistance. Therefore, the ability of MIC-1 to inhibit macrophage functions might counteract insulin resistance (159,160). Moreover, MIC-1 shows correlations with pro- and anti-inflammatory cytokines. It is negatively associated with the expression/release of pro-inflammatory mediators such as leptin and IL-1β in 3T3-L1 adipocytes. In human adipocytes, MIC-1 expression/release is positively associated with the anti-inflammatory mediator adiponectin (156). In addition, MIC-1 contributes to appetite and energy homoeostasis and could serve as potential target for the treatment of obesity (161). Overexpression of MIC-1 led to weight loss and decreased food intake in C57/BL6 mice (156). The WHS demonstrated that MIC-1 had cardioprotective effects (162). This is an interesting observation because CVDs are an important complication of type 2 diabetes.

In conclusion, MIC-1 shares several features with adiponectin such as the inverse correlation with obesity and its anti-inflammatory properties, but its specific role in type 2 diabetes is yet unclear.

1.2.4.4 Secreted frizzled-related protein 5 (Sfrp5)

Secreted frizzled-related protein 5 (Sfrp5) is a recently identified adipokine with anti-inflammatory characteristics. Sfrp5 belongs to the Sfrp family and is exclusively expressed in adipose tissue (163). Sfrp5 antagonises wingless-type MMTV integration site family, member 5a (Wnt5a) that acts as a pro-inflammatory mediator and stimulates the release of pro-inflammatory cytokines (164,165). In obese mice, Wnt5a levels are increased which causes an increase in the concentrations of IL-6 and TNFα and the amount of macrophages in adipose tissue. This pro-inflammatory activation can be inhibited by Sfrp5. Both, Sfrp5 and Wnt5a are involved in the non-canonical Wnt signalling which means that they act by activation of JNK instead of β-catenin (canonical Wnt signalling) (163).

So far, there is only one study on the association between Sfrp5 and obesity, inflammation and insulin resistance (163) that was performed in obese mice. In

these mouse models, Sfrp5 levels were released in lower amounts. Sfrp5-deficient mice fed a high-fat high-sucrose diet had higher fasting serum glucose and fasting serum insulin content, a higher body weight, higher liver triglyceride content, higher degree of hepatic steatosis and macrophage-mediated inflammation. The administration of recombinant Sfrp5 improved metabolic functions by increased glucose clearance and reduced hepatic steatosis as well as reduced adipose tissue inflammation (163).

Taken together, Sfrp5 shares several characteristics with adiponectin such as the exclusive expression in adipose tissue, lower production in obesity, anti-inflammatory properties and improvement of insulin resistance. However, the role of Sfrp5 in the development of type 2 diabetes is still unclear.

1.3 Epidemiological studies

A major aim of epidemiological studies is the identification of risk factors that contribute to the development of a disease. Risk factors can be of different nature such as environmental factors, genetic predisposition or behavioural characteristics. In epidemiological research several study types are used such as cohort studies, case-cohort studies and nested case-control studies (166).

Another important study type is the intervention study with the purpose of improving health or altering the course of disease (167).

1.3.1 Cohort study

In a cohort or longitudinal study, the participants are followed over time. At the beginning of a cohort study, the cohort (group of study participants) has not experienced the outcome of interest (e.g., manifestation of type 2 diabetes), but all participants are at risk. Each member of the cohort is classified according to those expositions (possible risk factors, e.g., systemic levels of immune mediators) that might be associated with the outcome. All members of the cohort are observed over time to determine who experiences the outcome and who does not. At the end of the study the association between outcome and risk factor can be analysed. The most important advantage of this study design is that the study participants are first exposed to the risk factor and then eventually experience the outcome. Therefore, an analysis of causality is possible.

There are two ways to perform a cohort study: (1) prospective cohort study: the cohort is assembled in the present and followed into the future; (2) retrospective cohort study: the cohort is identified from past records and is followed forward from that time up to the present (168).

If it is too expensive and/or there is not enough capacity to collect and process covariate information on all participants of the cohort study, it is possible to analyse only a part of this cohort. These variations of the cohort design are called *case-cohort study* and *nested case-control study*. Both study designs represent methods of sampling from an assembled epidemiological cohort study (169).

A *case-cohort study* is based on a random sample of the initial cohort, called the subcohort, which is compared to all cases that occur in the whole cohort. The size of the subcohort depends on the number of cases that were identified as

manifesting the disease during follow-up. Matching of cases and non-caeses is not performed. All members of the case-cohort study are members of the subcohort and all cases. Therefore, an overlap between those two sources is possible (169,170).

The *nested case-control study* is based on a random sampling from eligible controls (who do not experience the outcome at the time the corresponding cases do) for each case. In comparison with a case-cohort study, there is a pair matching in a nested case-control study (169).

1.3.2 Intervention study

The intervention study is a study design in which researchers actively intervene aiming to influence a certain outcome, e.g., to prevent type 2 diabetes by altering lifestyle habits of the study participants. The experimental group receives a specific treatment, whereas the control group gets a placebo or standard treatment. The distribution of the study participants into the two groups is performed randomly. If the study has a blinded design, the study participants do not know whether they belong to the intervention or control group. If the study is a double-blinded experiment, neither study participants nor experimenters know who belongs to the intervention or control group (167).

1.3.3 Confounders

There is one important problem in the analysis of the results of the aforementioned studies. The analysis of the association between outcome (e.g., type 2 diabetes) and exposition (possible risk factor, e.g., a certain immune mediator) can be influenced by confounders (another exposition, e.g., BMI). The confounder has several characteristics: it is associated with the outcome (but is not cause of the outcome) and with the investigated exposition and it can be associated with the outcome if the investigated exposition is not existent. There are several methods to deal with the potential influence of confounders: (a) randomisation to distribute the confounders equally between cases and controls, (b) matching for possible confounders (case-control studies), (c) limitation of the study population to certain subgroups, (d) stratification (separate analysis for certain subgroups) and (e) multivariate analysis (statistical models to adjust for certain confounders) (171).

1.3.4 Study designs in this project

The data underlying the present thesis are based on serum samples from two studies: Whitehall II study and Coffee Intervention study.

1.3.4.1 Whitehall II study

The Whitehall studies were named after the Whitehall Road in London (United Kingdom) where all study participants worked or still work in an office of the British Civil Service. The Whitehall II study started in 1985 and is on-going. At the beginning 14,120 middle-aged persons were invited of whom 73% (10,308) participated at the first phase. These 10,308 study participants comprised 6,895 men and 3,413 women. Follow-up examinations alternately contained medical examinations and questionnaires or only questionnaires and were performed in intervals of 2.5 years. In phase 2 (1989) to phase 9 (2009), 63-86% of the individuals recruited at baseline participated in these phases. The 10th phase was performed in 2011 and included screening and questionnaires (277 participants). The 11th phase is planned to start in February 2012. (172,173). Phase 3 (1991-1994) was the first phase which included a 75 g-OGTT. Therefore, this phase was used as baseline for our investigations on IL-1Ra, adiponectin and MIC-1.

For the measurement of the immune mediators IL-1Ra and adiponectin in human serum we designed a prospective case-cohort study within the Whitehall II study. We defined a random sample from the source population leading to a case-cohort of 2,810 non-diabetic individuals at baseline. During a follow-up period of 8.8 ± 3.4 years 335 subjects developed type 2 diabetes whereas 2,475 subjects remained diabetes-free. We used repeated measurements at up to three time-points: phase 3 (1991-1994), phase 5 (1997-1999) and phase 7 (2002-2004) to investigate the course of IL-1Ra and adiponectin levels before the manifestation of type 2 diabetes.

For the measurement of MIC-1 in human serum we designed a prospective, nested case-control study within the Whitehall II study. The selected study participants were diabetes-free at baseline (phase 3), and we used the serum samples from this phase to measure MIC-1 in the serum of the study participants. The number of selected individuals who developed type 2 diabetes during the follow-up period of 11.5 ± 3.0 years was 180, whereas 372 individuals with NGT at

baseline and during follow-up served as controls. Controls were frequency-matched to cases for age (5-year bands), sex and BMI (5 kg/m^2 bands).

1.3.4.2 Coffee Intervention study

The Coffee Intervention study is a single-blind (investigator), three-stage study that was performed in Finland. Coffee consumers were recruited by advertisements in newspapers. In this study, 47 predominantly obese individuals refrained from coffee drinking for one month. In the second month, they consumed four and in the third month eight cups of coffee per day (1 cup = 150 ml). Serum samples were collected at the end of each month before and after an OGTT. The study participants were younger than 69 years and free of type 2 diabetes, but with an increased risk of type 2 diabetes (i.e., 13 or more than 13 points in the Finnish risk score) (174). Serum samples were collected for various measurements which included the analyses of inflammation-related biomarkers at the German Diabetes Center. These serum samples were used for the measurement of Sfrp5.

1.4 Objectives

Subclinical inflammation is one important mechanism contributing to the development of type 2 diabetes. It is characterised by chronically altered levels of expression and release of immune mediators that are involved in the induction of insulin resistance and β-cell failure. Data from numerous studies on the association of pro-inflammatory immune mediators with type 2 diabetes are available. IL-1β is one pro-inflammatory immune mediator that promotes the development of type 2 diabetes. Thus, IL-1β has been used as therapeutic target to block its deleterious effects on insulin-responsive tissues and β-cells and to treat type 2 diabetes. Serum levels of IL-1β are usually below the detection limit of currently available assays, so that its measurement in epidemiological studies is not feasible.

In contrast to pro-inflammatory immune mediators, the relevance of anti-inflammatory immune mediators in the development of type 2 diabetes is poorly characterised. In particular, data on changes of their circulating levels in relation of incident type 2 diabetes are not available. Therefore, we decided to analyse serum levels of the naturally occurring IL-1β antagonist, the anti-inflammatory protein IL-1 receptor antagonist (IL-1Ra) before the diagnosis of type 2 diabetes. Moreover, in this analysis we also investigated adiponectin, the only anti-inflammatory immune mediator for which high levels are described to be associated with a reduced risk of type 2 diabetes. Because of the putative diabetes-protective properties of adiponectin we also included the novel immune mediators MIC-1 and Sfrp5 which have not yet been analysed with respect to the risk of type 2 diabetes and whose regulation shows similar features as that of adiponectin.

This doctoral thesis focused on three main aims:

(1) The first aim was to characterise the time-course of serum levels of IL-1Ra and adiponectin before the manifestation of type 2 diabetes. The Whitehall II cohort was selected for this investigation because serum samples as well as OGTT data were available from different time-points for each study participant. So far, most investigations of immune mediators were based on only one baseline measurement of these immune mediators in prospective studies. Analyses based on single biomarker measurements are essential to elucidate the association between the measured immune mediator and

incident type 2 diabetes, but give only little information on the natural history of type 2 diabetes. Until now, data describing the time-course of the concentrations of immune mediators before the manifestation of type 2 diabetes are not available.

We tested the hypothesis that IL-1Ra levels increase about 5 years before the manifestation of type 2 diabetes because a previous investigation demonstrated that insulin sensitivity and β-cell function change during this period of time. We expected that changes in serum levels of immune mediators are associated with metabolic changes before type 2 diabetes manifestation.

In contrast to IL-1Ra, adiponectin was assumed to decrease before the diagnosis of type 2 diabetes because increased levels of adiponectin were described to be associated with a reduced risk of type 2 diabetes and to be protective against type 2 diabetes.

(2) The second aim was to investigate the association between MIC-1 and the risk of incident type 2 diabetes for the first time. This analysis was based on a smaller selection of baseline serum samples also used for the investigations on IL-1Ra and adiponectin (Whitehall II study).

As the effects of MIC-1 resemble adiponectin effects in some features, we expected that increased levels of MIC-1 are associated with a reduced risk of type 2 diabetes as described for adiponectin.

(3) The third aim was to characterise circulating levels of Sfrp5 in human serum for the first time. For this analysis, we used serum samples from study participants of an intervention study.

Because Sfrp5 shared several features with adiponectin in obese mice we hypothesised that associations of Sfrp5 with anthropometric and metabolic parameters would be similar in human serum as described for adiponectin.

Chapter 2

Accelerated Increase in Serum Interleukin-1 Receptor Antagonist Starts 6 Years Before Diagnosis of Type 2 Diabetes Whitehall II Prospective Cohort Study

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OBJECTIVE—Although interleukin-1 receptor antagonist (IL-1Ra) treatment is associated with improved β-cell function and glycemic control in patients with type 2 diabetes, its role in the development of type 2 diabetes remains unclear. We used repeated measurements to characterize IL-1Ra trajectories in individuals who developed type 2 diabetes.

RESEARCH DESIGN AND METHODS—This case-cohort study, nested within the Whitehall II cohort, was based on 335 incident type 2 diabetes cases and 2,475 noncases. We measured serum IL-1Ra levels at up to three time points per individual and estimated retrospective trajectories of IL-1Ra before diabetes diagnosis (case subjects) or end of follow-up (control subjects) using multilevel analysis. Models were adjusted for age, sex, and ethnicity.

RESULTS—IL-1Ra levels were already higher in the case than control subjects 13 years before diabetes diagnosis/end of follow-up (mean [95% CI] 302 [290–314] vs. 244 [238–249] pg/ml). In control subjects, IL-1Ra levels showed a modest linear increase throughout the study period. In case subjects, IL-1Ra trajectories were parallel to those in control subjects until 6 years (95% CI 7.5–4.5) before diagnosis and then rose steeply to 399 (379–420) pg/ml at the time of diagnosis ($P < 0.0001$ for slope difference). Adjustment for BMI and waist circumference as time-varying covariates had little impact on these trajectories.

CONCLUSIONS—We show elevated IL-1Ra levels for 13 years and an accelerated increase during the last 6 years before type 2 diabetes diagnosis, indicating the presence of an anti-inflammatory response that may act to counterbalance the metabolic and immunologic disturbances that precede type 2 diabetes. ***Diabetes*** **59:1222–1227, 2010**

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Type 2 diabetes is characterized by insulin resistance and impaired insulin secretion, the latter being due to a reduction in β-cell mass and an increase in β-cell apoptosis (1). Prospective cohort studies have shown elevated circulating levels of acute-phase proteins, cytokines, and chemokines to predict incident type 2 diabetes (2–6). Interleukin-1β (IL-1β) may be one of the most important immune mediators in this context because this cytokine triggers various proinflammatory events, inhibits β-cell function, and promotes β-cell apoptosis (7,8). The deleterious effects of IL-1β can be inhibited by its naturally occurring antagonist, IL-1 receptor antagonist (IL-1Ra), produced by adipose and other tissues (9). IL-1Ra competitively binds to the IL-1 receptor without inducing a cellular response (7,10,11).

Animal models and evidence from humans support the association between IL-1Ra and diabetes. In mice, IL-1Ra injections have been demonstrated to protect from diabetes induced by high-fat diet (12). A double-blind, parallel-group trial of 70 patients with type 2 diabetes showed that the administration of exogenous IL-1Ra (anakinra) improved glycemia, β-cell function, and surrogate markers of systemic inflammation (13,14).

A nested case-control study within the Whitehall II study found that high levels of IL-1Ra were associated with an increased risk of incident type 2 diabetes. This finding suggests that increased IL-1Ra levels may reflect a reaction to counterregulate immunologic and metabolic disturbances before the onset of type 2 diabetes—a reaction that eventually fails (15).

Recent advances in analysis of longitudinal risk factor trajectories support a multistage model of diabetes development. Although the levels of homeostasis model assessment insulin sensitivity (HOMA-2S) and HOMA β-cell function (HOMA-2B) differed between incident cases and noncases more than a decade before the diagnosis of type 2 diabetes, a substantial divergence in HOMA trajectories, accompanied by a marked acceleration in the dysregulation of glucose metabolism, became evident during the last 5 years before diagnosis (16). Extending the risk factor trajectory approach to inflammatory biomarkers could improve our understanding of the pathogenesis of type 2 diabetes by providing insight into inflammatory changes that may be causally related to disease onset (17).

In this report from the Whitehall II study, we examine the trajectories of serum IL-1Ra in individuals who develop type 2 diabetes and those who remained diabetes-

free. We hypothesize that an accelerated increase in circulating IL-1Ra levels would precede diabetes development by ~5 years before the diagnosis, coinciding with the previously described changes in insulin sensitivity and β-cell function (16). Changes in IL-1Ra would indicate an anti-inflammatory reaction to counterregulate the proinflammatory environment and preserve insulin sensitivity and β-cell function.

RESEARCH DESIGN AND METHODS

Study population. Data are from a nested case-cohort study within the prospective Whitehall II cohort study of 10,308 British civil servants aged 35–55 years at phase 1 (1985–1988). Details regarding study design, baseline characteristics, and source population for the case-cohort study have been described previously (18,19). The study was approved by the University College London Medical School Committee on the Ethics of Human Research and conducted according to the Declaration of Helsinki. Written informed consent was obtained at baseline and renewed at each contact.

Phase 3 (1991–1994), when a 75-g oral glucose tolerance test (OGTT) was administered for the first time, served as baseline for the current study. Participants were followed through postal questionnaires at 2.5-year intervals (phase 4: 1995–1996, phase 6: 2001, phase 8: 2006–2007) and through clinical examinations including an OGTT at phase 5 (1997–1999) and phase 7 (2003–2004). For the case-cohort study, we drew a random sample from the source population of 6,058 men and 2,758 women who had attended the phase 3 examination. We excluded participants with prevalent type 2 diabetes at phase 3 (n = 42), missing follow-up data on diabetes (n = 552), or missing data for key variables (C-reactive protein [CRP; limited to subjects with CRP <10 mg/l], weight, waist circumference, cholesterol, triglycerides, fasting glucose, fasting insulin) at baseline (n = 2,018) or during follow-up (phases 5 and 7; n = 3,049), leading to a case cohort of 2,810 subjects: 335 subjects with incident type 2 diabetes and 2,475 subjects without incident type 2 diabetes.

Measurements. Diabetes was defined by a fasting glucose of 7.0 mmol/l or more, or a 2-h postload glucose of 11.1 mmol/l or more (20). Type 2 diabetes was diagnosed by OGTT (56.4%), self-report (13.1%), or the use of glucose-lowering medication (30.4%).

We measured blood glucose with the glucose oxidase method on a YSI model 23A glucose analyzer (phase 3: mean coefficient of variation [CV] 2.9–3.3%) and YSI model 2300 STAT PLUS analyzer (phases 5 and 7: mean CV 1.4–3.1%) (YSI, Yellow Springs, OH) (16). Serum insulin was determined with an in-house human insulin radioimmunoassay (phase 3: mean CV 7%) and a DAKO insulin ELISA kit (Dako Cytomation, Ely, U.K.) (phases 5 and 7: mean CV 4.2–9.3%) (16).

IL-1Ra serum concentrations were measured with the Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany). Although serum samples were from three different study phases, blood collection, processing, and storage at −80°C followed the same standard operating procedures. All assays were performed consecutively in the same laboratory (German Diabetes Center), and samples from different study phases of the same study participant were always measured using the same enzyme-linked immunosorbent assay (ELISA) plate to minimize assay imprecision. Mean intra-assay and interassay CVs were 2.6 and 7.9%, respectively. The limit of detection was 14 pg/ml. All samples gave values above the limit of detection.

The following variables were included as time-invariant covariates: sex, age at the end of follow-up, and ethnicity (0 = white, 1 = nonwhite). Data for these were derived from phase 3 and phase 1 questionnaires. Given that adipose tissue is a major producer of IL-1Ra (9), BMI and waist circumference (assessed in medical examinations contemporaneously with IL-1Ra measurements) were included as additional covariates and were coded as time-varying covariates.

Statistical analysis. Statistical analyses were performed using SPSS 14.0 statistical software (SPSS, Chicago, IL). The participants were divided into case subjects (i.e., individuals who developed type 2 diabetes during the follow-up) and control subjects (i.e., people who remained diabetes-free). Initial analyses compared the characteristics of case and control subjects and tested the differences using t tests and χ^2 tests. The unadjusted associations between IL-1Ra and potential confounding variables were examined and tested using ANOVA for stratified measures and Spearman rank correlation together with unstandardized regression coefficients (95% CIs) of $\log_2$(IL-1Ra) for the continuous variables. For subsequent analyses, year 0 of the observation period was the year of diabetes diagnosis for case subjects and a randomly selected time point during follow-up for control subjects to approximate the follow-up time distribution of case subjects. IL-1Ra levels were traced backwards to participants' first participation in clinical screening (i.e., phase 3, which represents the baseline of the current analysis—a maximum of 13 years previously). Multilevel models were fitted to the data to assess changes in IL-1Ra during these preceding 13 years (21). Of a total of 8,233 serum measurements (964 in case subjects; 7,269 in control subjects), 2,383 measurements after year 0 were excluded and thus the analyses were based on 755 serum samples in case subjects and 5,095 serum samples in control subjects. Data were structured so that measurement times (observations) were nested within subjects, and the standard errors were calculated by taking into account the nonindependence of the observations, that is, that the same individuals contributed to more than one observation in the dataset. We treated observation time as one period (a nonpiecewise approach) for control subjects and as two distinct periods (a piecewise approach) for case subjects. In the latter approach, we created two time variables: a continuous variable, scaled to take the value zero at the start of the second period, and a dummy variable indicating the period (0 = first period and 1 = second period). We first established the most parsimonious model for each piecewise model and chose the position of the start of the second period (from −9 to 0) that had the lowest information criteria for the final model. We estimated the likelihood-based 95% CI for the position of the start of the second period. The selected model was in close agreement with locally weighted scatter plot smoothers displaying the association graphically (data not shown). All analyses were adjusted for age, sex, ethnicity, and study phase. Additional models included BMI, waist circumference, or insulin as time-varying covariates. To provide figures adjusted for baseline characteristics, trajectories were fitted for a hypothetical population of 72.0% male and 91.2% white at age 62.9 years at the end of follow-up. Statistical significance was inferred at a two-tailed $P < 0.05$.

TABLE 1
Characteristics of incident diabetes case and control subjects at baseline (phase 3)

	Control subjects	Case subjects	P
n	2,475	335	
Age (years)	49.1 ± 5.8	51.0 ± 6.1	<0.0001
BMI (kg/m^2)	25.0 ± 3.3	27.1 ± 4.2	<0.0001
Waist circumference (cm)	83.5 ± 10.8	89.2 ± 12.2	<0.0001
Systolic blood pressure (mmHg)	119.5 ± 12.7	123.8 ± 14.3	<0.0001
Diastolic blood pressure (mmHg)	79.3 ± 9.0	82.2 ± 9.6	<0.0001
Fasting blood glucose (mmol/l)	5.2 ± 0.4	5.5 ± 0.5	<0.0001
2-h blood glucose (mmol/l)	5.3 ± 1.4	6.7 ± 1.9	<0.0001
Fasting insulin (pmol/l)	36.2 ± 28.8	61.5 ± 46.4	<0.0001
Sex, male/female (%)	73.3/26.7	69.9/30.1	0.191
Ethnicity, white/nonwhite (%)	92.9/7.1	80.6/19.4	<0.0001

Data are means ± SD or %.

RESULTS

Study population. The comparison of the study participants (n = 2,810) and excluded subjects (n = 6,006) at baseline showed the study participants to be, on average, 1.4 years younger than those excluded, but otherwise revealed only small differences between the groups (supplementary Table 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1199/DC1). Characteristics of case and control subjects at baseline (phase 3) are shown in Table 1. Case subjects (n = 335) were older and more overweight than control subjects (n = 2,475). Case subjects also had higher blood pressure, fasting and 2-h blood glucose, and fasting insulin and were more likely to be nonwhite. Mean follow-up time (± SD) was 8.8 ± 3.4 years.

Determinants of serum concentrations of IL-1Ra (univariate analyses). Unadjusted baseline levels of IL-1Ra were higher in case than control subjects (Table 2).

TABLE 2
IL-1Ra levels in subgroups of the study population at baseline (phase 3)

Stratification variable	IL-1Ra (pg/ml)	*P*
Diabetes incidence		
Cases	308 (293–323)	
Noncases	248 (244–252)	<0.0001
Sex		
Male	246 (242–251)	
Female	278 (269–287)	<0.0001
Ethnicity		
White	255 (251–259)	
Nonwhite	253 (238–268)	0.78

Data are means (95% CI).

In addition, IL-1Ra levels were higher in women than men. No differences in IL-1Ra were found among ethnic groups. In univariate analyses, IL-1Ra was positively correlated with age, BMI, waist circumference, blood pressure, 2-h glucose, and fasting insulin (Table 3).

Trajectories of IL-1Ra in case and control subjects. Multilevel models adjusted for age, sex, and ethnicity showed that serum IL-1Ra levels were already higher in case than control subjects 13 years before diagnosis or end of follow-up. Mean IL-1Ra levels (95% CI) were 302 (290–314) pg/ml in case subjects and 244 (238–249) pg/ml in noncase subjects (Fig. 1*A*).

From years −13 to −6 (95% CI −4.5 to −7.5), the trajectories of IL-1Ra of case and control subjects were parallel and increased marginally (<1.5 pg/ml per year) over time. An interaction effect among caseness, time period, and time indicated that the IL-1Ra trajectories of case and control subjects began to separate after year −6 (Fig. 1*A*). From years −6 to 0, IL-1Ra levels in case subjects increased steeply (13–16 pg/ml per year) and reached 399 (379–420) pg/ml at the time of diagnosis, whereas the slope of the IL-1Ra trajectory in the control subjects remained unaltered ($P < 0.0001$ for the slope difference during the final 6 years; model 1 in Table 4).

Additional adjustment for BMI or waist circumference as time-varying covariates reduced, but did not remove, the differences between case and control subjects (Fig. 1*B* and *C*). From years −13 until −6, IL-1Ra levels remained constant in both case and control subjects after adjustment. From years −6 until 0, IL-1Ra levels in case subjects increased steeply (~11–12 pg/ml per year in BMI-adjusted and 9–11 in waist-adjusted models), whereas IL-1Ra levels in control subjects remained unaltered ($P < 0.001$ for the slope difference during the final 6 years; models 2–3 in Table 4). Adjustment for fasting insulin had hardly any impact on the difference between case and control subjects from years −13 to −6, but substantially reduced the increase of IL-1Ra from years −6 to 0 in case subjects (~4 pg/ml per year; $P < 0.05$ for the slope difference during the final 6 years; model 4 in Table 4, Fig. 1*D*).

Estimated trajectories for fasting glucose (supplementary Fig. 1*A*), 2-h glucose (supplementary Fig. 1*B*), HOMA insulin sensitivity (supplementary Fig. 1*C*), and HOMA β-cell function (supplementary Fig. 1*D*) are provided for direct comparisons with the IL-1Ra trajectories. Apart from an earlier start of the compensatory increase in β-cell function in the current analysis (year −5 vs. −4), the shapes of the trajectories are almost identical to those observed in the larger Whitehall II cohort (16).

DISCUSSION

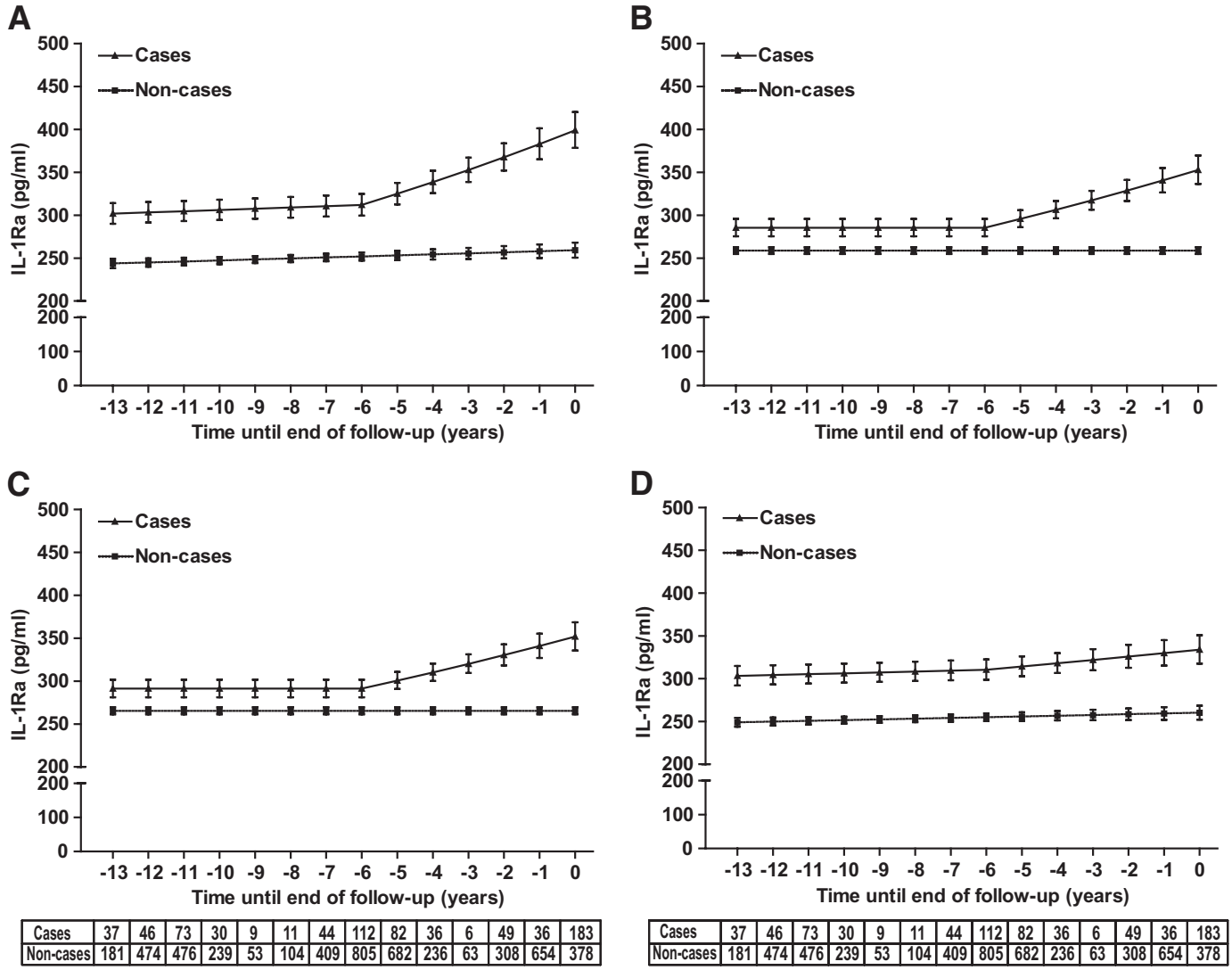
This case-cohort study of a middle-aged metabolically healthy population at baseline has three main findings. First, circulating concentrations of IL-1Ra were elevated in cases of incident type 2 diabetes 13 years in advance of diagnosis compared with individuals who remained diabetes-free. Second, in control subjects, longitudinal changes could be described by a linear trajectory with only a slight increase over time, whereas in case subjects, IL-1Ra increased rapidly starting 6 (95% CI 4.5–7.5) years before diagnosis. Third, changes in obesity explained the slight linear increase in control subjects throughout the observation period and in case subjects up to 6 years before diabetes diagnosis, but did not account for the steep increase in the IL-1Ra trajectory among case subjects in the last 6 years.

Our findings extend current knowledge on the association between inflammation and type 2 diabetes development, as this is the first study to characterize cytokine trajectories before type 2 diabetes. Our previous report on IL-1Ra and incident diabetes in a nested case-control study was limited to a single IL-1Ra measurement at study baseline (phase 3) (15) and therefore did not provide information on the time course of IL-1Ra levels preceding diabetes diagnosis.

Our findings are also novel from a pathophysiological perspective because they support the hypothesis that the pre-diabetic stage is characterized not only by proinflammatory alterations, but also by the presence of an anti-inflammatory response. Several mechanisms could explain the upregulation of IL-1Ra before the diagnosis of type 2 diabetes. The steep increase of IL-1Ra in pre-diabetic individuals occurs within the same time window in which indicators of insulin sensitivity, β-cell function, and glycemia deteriorate (supplementary Fig. 1). This may indicate that these unfavorable changes in glucose metabolism and the increase of IL-1Ra production are closely connected.

TABLE 3
Nonparametric correlations and parametric regression coefficients for the association of anthropometric and metabolic factors with IL-1Ra (log_2) at baseline (phase 3)

	Spearman *r*	*P*	β (95% CI)	*P*
Age (years)	0.066	0.0005	0.007 (0.003–0.011)	0.0004
BMI (kg/m^2)	0.370	<0.0001	0.070 (0.065–0.076)	<0.0001
Waist circumference (cm)	0.278	<0.0001	0.016 (0.014–0.018)	<0.0001
Systolic blood pressure (mmHg)	0.075	0.0001	0.003 (0.001–0.005)	0.0005
Diastolic blood pressure (mmHg)	0.104	<0.0001	0.007 (0.004–0.009)	<0.0001
Fasting blood glucose (mmol/l)	0.035	0.063	0.079 (0.030–0.128)	0.0016
2-h blood glucose (mmol/l)	0.210	<0.0001	0.086 (0.071–0.100)	<0.0001
Fasting insulin (mmol/l)	0.283	<0.0001	0.005 (0.005–0.006)	<0.0001



Cases	37	46	73	30	9	11	44	112	82	36	6	49	36	183
Non-cases	181	474	476	239	53	104	409	805	682	236	63	308	654	378

FIG. 1. *A–D*: Model-predicted IL-1Ra trajectories in nondiabetic and incident diabetic subjects. Year 0 denotes the time of diagnosis for case subjects and a randomly selected time point during follow-up for control subjects (noncases). All models were adjusted for age, sex, and ethnicity. Additional adjustment was performed for BMI (*B*), waist circumference (*C*), and fasting insulin (*D*). The table below the panels shows the number of measurements at each time point for both case and control subjects. Error bars represent 95% CIs around the values estimated by the fixed effect part of the mixed model presented in Table 4.

In vitro studies show that both human islets and monocytes respond to high glucose concentrations with an upregulation of IL-1β (22–24), so that increased IL-1Ra concentrations before type 2 diabetes may represent a response to glucose-mediated IL-1β upregulation. The balance between IL-1β and IL-1Ra has been postulated to be a major determinant of the time course and severity of inflammatory diseases (25,26), and it is conceivable that

TABLE 4
Fixed effects for multilevel models of changes over time in $\log_2$(IL-1Ra) serum concentrations before diagnosis of type 2 diabetes or the end of follow-up

	Model 1	Model 2	Model 3	Model 4
Time (per year)	0.004 (0.002)*	NS	NS	0.005 (0.002)*
Case (incident type 2 diabetes)	0.31 (0.03)†	0.14 (0.03)†	0.13 (0.03)†	0.28 (0.029)†
Case × time	NS	NS	NS	NS
Case × time × 2nd period	0.052 (0.006)†	0.051 (0.006)†	0.045 (0.006)†	0.013 (0.006)*
BMI (per kg/m^2)	—	0.065 (0.002)†	—	—
Waist circumference (per cm)	—	—	0.025 (0.001)†	—

Data are regression coefficients (SE). Time = continuous variable scaled so that time = 0 at 6 years before diagnosis or the end of follow-up. Case = incident type 2 diabetes case. 2nd period = dummy variable (1 for positive values in the time variable, i.e., later than 6 years before diagnosis or the end of follow-up; 0 for nonpositive values). Trajectories in 335 case subjects with incident type 2 diabetes were compared with those in 2,475 control subjects. $\log_2$(IL-1Ra) was the outcome variable of the multilevel longitudinal modeling, and data were adjusted for age, sex, ethnicity, and study phase. Only models with the lowest information criteria are shown. *$P < 0.05$; †$P < 0.0001$.

the local and/or systemic ratio of these cytokines could also be relevant in the pathogenesis of type 2 diabetes. We could not test this hypothesis here because the physiological concentrations of IL-1β in individuals without severe inflammatory diseases are so low that they are mostly undetectable with currently available assays (23,27). However, it has been suggested that elevation of only IL-1β may not be sufficient to increase risk of type 2 diabetes; instead, increased IL-1β in combination with elevated levels of other proinflammatory cytokines may be required (28), thus limiting the predictive relevance of the IL-1Ra/IL-1β ratio without consideration of other risk factors.

The finding that the difference in trajectories between case and control subjects could not be explained by obesity is important because adipose tissue is a major producer of IL-1Ra (9,29). We replicated strong correlations of IL-1Ra levels with BMI and waist circumference (30–33). However, inclusion of BMI or waist circumference as time-varying covariates had no major effect on the shape of IL-1Ra trajectories, suggesting that the upregulation of IL-1Ra cannot directly be attributed to weight gain. Differences in secretion of leptin, an adipokine strongly upregulated in obesity that can stimulate IL-1β production and lead to increased IL-1Ra release, may be one of the mechanisms involved (34,35).

We did not adjust for glycemia, because our stratifying variable (incident diabetes caseness) already includes fasting and/or postload glucose in its definition, which means that we have already adjusted for glucose values at time 0. Because fasting insulin is not included in the diagnostic criteria, we performed an analysis adjusted for fasting insulin, which substantially attenuated the slope difference between case and control subjects preceding the diagnosis of diabetes. However, a significant acceleration of the slope from years −6 to 0 remains among case subjects, indicating that fasting insulin explains or mediates some, but not all, of the late increase in IL-1Ra. It should be kept in mind that with our study design, we cannot directly establish whether this attenuation is due to confounding, mediation, or shared causation.

Our findings point to the possibility that shape of biomarker trajectories is informative in terms of assessing their predictive value for different time windows. Sequential measurements of cytokines and other biomarkers and the characterization of individual trajectories may improve the estimation of type 2 diabetes risk. Differences in IL-1Ra levels between case and control subjects varied considerably over the lead time such that IL-1Ra may be more useful in predicting short-term diabetes risk, whereas other cytokines may be more strongly associated with long-term risk of type 2 diabetes. To date, attempts to improve diabetes risk prediction in the general population based on biomarker panels measured at a single time point have produced only marginal improvements compared with conventional risk models. It remains to be determined whether it will be possible to improve risk prediction at different pre-diabetic stages in the general population by considering serial biomarker measurements over time.

From a therapeutic point of view, it is noteworthy that recombinant IL-1Ra has been shown to improve metabolic control in patients with type 2 diabetes (13,14). In this light, an upregulation of IL-1Ra may be expected to be protective rather than associated with increased risk. In our study, it appears that the steep increase of IL-1Ra levels by approximately one-third was by far not sufficient to prevent the onset of type 2 diabetes. In the anakinra trial, an improvement of glycemia and β-cell function was accompanied by supraphysiological IL-1Ra peak levels in serum that were >1,000-fold higher in the intervention group than in the placebo group (13).

Our study has some limitations and strengths that should be acknowledged. First, the study design does not enable us to exclude the alternative interpretation that the increase of IL-1Ra before type 2 diabetes contributes to the disease risk and represents a causal factor rather than an anti-inflammatory response. However, data from the anakinra trial (13) as well as preclinical data (12,36) argue strongly against diabetogenic effects of IL-1Ra. Second, we did not determine IL-1β levels and therefore cannot know whether IL-1β increases before type 2 diabetes and whether such an increase precedes the increase in IL-1Ra. In addition, we did not have data from all three study phases for other proinflammatory markers, such as CRP and IL-6, for a comparison of trajectories. We did not adjust for the available CRP or IL-6 levels at baseline, because this adjustment has the capacity to influence only the intercept of the model, not the shape of the trajectories, and therefore would not help to answer the question of temporal sequences. Third, the Whitehall II study is an occupational cohort and as such is not population based. Although the cohort was healthy at recruitment, as in a community-based sample, the cohort overrepresents white, male, and middle-aged individuals. Fourth, due to missing data, approximately two-thirds of the source population at phase 3 was excluded from the present analysis. However, more than 300 incident cases and a total of almost 6,000 measurement points for the trajectories led to sufficient statistical power to detect differences between case and control subjects. In addition, our dropout analysis (supplementary Table 1) indicates that exclusions are unlikely to have affected internal validity.

The study has notable strengths. It is based on a well-phenotyped cohort, and more than half of incident diabetes cases were diagnosed based on the gold standard oral glucose tolerance test. We applied a sophisticated methodology that considered the interrelation among repeated measurements from the same individual at different time points during the follow-up and based our analysis on a large sample, as noted above.

In conclusion, we characterized cytokine trajectories to understand the evolution of the association between IL-1Ra levels in the circulation and type 2 diabetes before the diagnosis of diabetes. IL-1Ra levels showed an accelerated increase during the last 6 years preceding diagnosis. We showed that crude measures of adiposity did not explain the IL-1Ra trajectory, potentially implicating other factors in disease susceptibility. These data support the hypothesis that an anti-inflammatory response counterbalances metabolic and immunologic disturbances preceding type 2 diabetes. Moreover, these results suggest that multiple measurements of inflammation-related and other biomarkers can markedly improve our understanding of the pathogenesis of type 2 diabetes. The clear temporal characterization shown by our data may help define the optimal clinical use of these biomarkers.

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Online-only appendix

Accelerated increase in serum interleukin-1 receptor antagonist (IL-1Ra) starts 6 years before diagnosis of type 2 diabetes: Whitehall II prospective cohort study

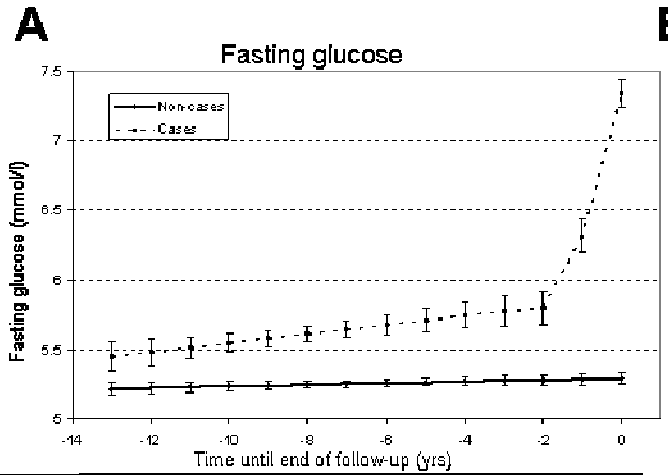
Maren Carstensen, Christian Herder, Mika Kivimäki, Markus Jokela, Michael Roden, Martin J. Shipley, Daniel R. Witte, Eric J. Brunner, Adam G. Tabák

Table A
Characteristics of study participants and excluded subjects at baseline (phase 3).

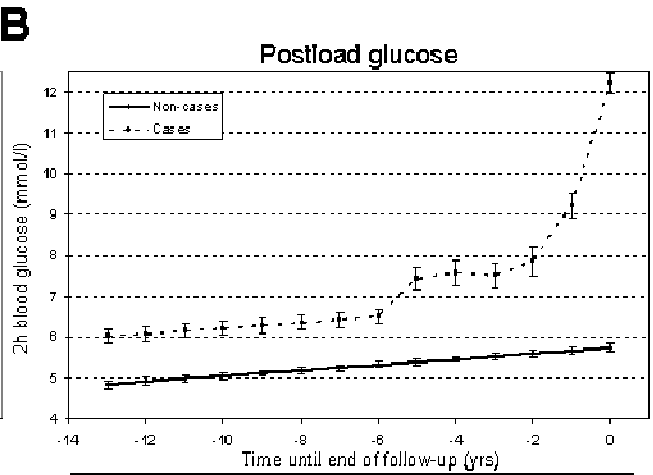
Variable	Participants (N = 2810)	Non-participants (N = 6006)	*P*
Age (years)	49.3 (5.9)	50.7 (6.1)	< 0.0001
BMI (kg/m^2)	25.3 (3.5)	25.3 (3.8)	0.328
Waist circumference (cm)	84.2 (11.2)	83.6 (11.7)	0.041
Systolic blood pressure (mmHg)	120.0 (13.0)	121.1 (13.9)	0.001
Diastolic blood pressure (mmHg)	79.6 (9.1)	79.8 (9.6)	0.525
Fasting blood glucose (mmol/l)	5.2 (0.5)	5.3 (0.8)	0.001
2 h blood glucose (mmol/l)	5.5 (1.5)	5.7 (2.2)	< 0.0001
Fasting insulin (pmol/l)	39.3 (32.5)	43.6 (38.8)	< 0.0001
Sex (%) Male / female	72.8 / 27.2	66.8 / 33.2	< 0.0001

Data are presented as mean (SD) or %.

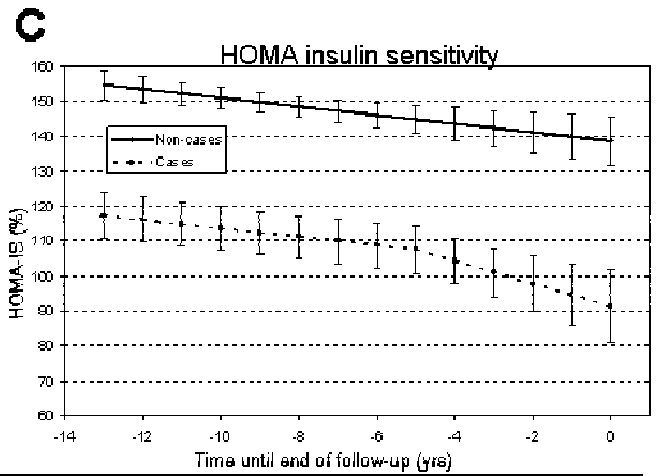
Fig. A. Multilevel longitudinal modelling using a linear growth model for non-diabetic and incident diabetic subjects with fasting glucose (A), 2-hour postload glucose (B), homeostasis model assessment (HOMA) insulin sensitivity (C), and HOMA beta-cell function (D) as outcomes. The year 0 is the time of diagnosis (cases) and a randomly selected time-point during follow-up (non-cases), respectively. Adjusted for age, sex, ethnicity and study phase. The table shows the number of measurements at each timepoint for both cases and non-cases. Error bars represent 95% confidence intervals around the values estimated by the fixed effect part of the respective mixed model.



Control	124	269	249	121	31	60	275	535	446	161	46	212	458	290
Inc. diabetes	35	40	51	32	10	13	46	97	73	36	4	45	31	123



124	269	249	121	31	60	267	522	432	155	44	210	453	266
35	40	51	32	10	13	42	97	70	34	3	43	30	122



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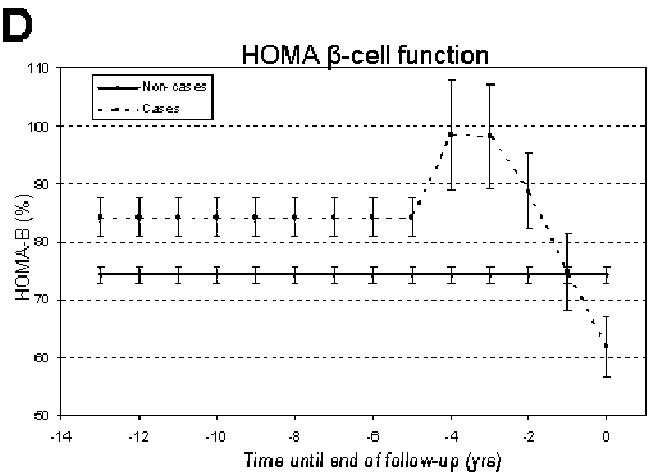

124	269	249	121	31	60	275	535	446	161	46	212	458	290
35	40	51	32	10	13	46	97	73	36	4	45	31	123

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Contribution: Total: 70%

Conceived / designed experiments:	40%
Performed experiments:	100%
Analysed data:	30%
Contributed to discussion:	40%
Wrote the manuscript:	50%
Reviewed / edited manuscript:	25%

Author: 1st authorship shared with PD Dr. Christian Herder

Chapter 3

Adiponectin Trajectories Before Type 2 Diabetes Diagnosis – Whitehall II study -

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Diabetes Care (in revision)

Adiponectin Trajectories Before Type 2 Diabetes Diagnosis – Whitehall II Study

Short running title: Adiponectin trajectories and type 2 diabetes

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Abstract

Objective – The role of adiponectin in the natural history of diabetes is not well characterized. We set out to characterize pre-diagnosis trajectories of adiponectin in individuals who develop type 2 diabetes.

Research Design and Methods – In a case-cohort study (335 incident diabetes cases, 2474 non-cases) nested in the Whitehall II study serum adiponectin was measured up to 3 times per participant (1991-1993, 1997-1999, 2003-2004). Multilevel models adjusted for age and ethnicity were fitted to assess 13-year trajectories of log-transformed adiponectin preceding diabetes diagnosis or a randomly selected time point during follow-up ($year_0$) based on 755/5095 (cases/non-cases) person-examinations.

Results – Adiponectin levels were lower in diabetes cases than non-cases (median [IQR] 7141 [5187-10,304] vs. 8818 [6535-12,369] ng/ml at baseline, $p<0.0001$). Controls showed a modest decline in adiponectin throughout follow-up (0.3%/year, $p<0.0001$), with higher levels in women than men (difference at $year_0$: 5358 ng/ml, $p<0.0001$). Female cases similarly to early-onset cases (age at diagnosis <52 years) had a steeper decline than controls (slope difference -1.1%/year, $p=0.001$ in females, -1.6%/year in early-onset cases, $p=0.034$). In men, adiponectin slopes for cases and non-cases were parallel. The slope differences by diabetes-onset were attenuated after adjustment for changes in obesity, whereas sex-specific slope differences were independent of obesity.

Conclusions – Lower adiponectin levels were observed already a decade before the diagnosis of diabetes. The marked sex difference in trajectories suggests that sex-specific mechanisms affect the association between adiponectin levels and diabetes development.

Adiponectin is an adipose-tissue-derived insulin-sensitizer. Adiponectin modifies glucose homeostasis and exhibits anti-inflammatory and anti-atherogenic effects.(1,2) Epidemiological data link lower adiponectin levels to disease states including type 2 diabetes, metabolic syndrome, hypertension, cardiovascular disease, and cancer.(3)

Insulin resistance is one of the major pathophysiological factors of diabetes, and adiponectin given its insulin-sensitizing effect may be centrally involved in the events leading to diabetes.(3-5) This is supported by the fact that adiponectin has independently predicted diabetes mellitus in longitudinal studies.(6-16)

Time-to-event analysis based on single biomarker measurements is essential for individual risk prediction and public health planning, but gives limited information on the natural history of a given disease. To provide new insights into the pathophysiology of diabetes, we utilized repeated measures of diabetes-related variables and described trajectories of glycemia and interleukin-1 receptor antagonist before diabetes diagnosis.(5,17) However, studies with repeat data on adiponectin in relation to diabetes development are scarce.(10,18-21) In spontaneously diabetic Rhesus monkeys, adiponectin trajectories until diabetes manifestation were declining.(20) Human studies (based on 2 measurement points per individual) have suggested decreasing adiponectin to be associated with an increase in insulin resistance or obesity.(19,21) Diabetes prevention trials reported increasing adiponectin levels in intervention groups with parallel weight loss.(10,18)

To overcome the limitations of the previous studies (i.e., lack of well-defined incident diabetes and control groups, insufficient number of repeat measures), we conducted up to 3 clinical examinations per individual to investigate adiponectin trajectories in a middle-aged British population separately among people who

developed incident diabetes and those who remained normoglycemic during follow-up. In addition to adjustments for age and ethnicity, we took into account factors related to insulin resistance, such as sex, age at onset of diabetes, and obesity.

RESEARCH DESIGN AND METHODS

Study Population and Design

We present results from a nested case-cohort study within the Whitehall II prospective cohort. The cohort was established between 1985-1988 (phase 1) and included 10,308 (6895 men) non-industrial British civil servants aged 35-55 years working in London offices of 20 departments.(22,23) Study phase 3 (1991-1993) when glucose tolerance was first assessed by a 75g oral glucose tolerance test (OGTT) serves as the baseline for the current analysis (men/women: n=6058/2758). Participants were followed through postal questionnaires at approximately 2.5-year intervals (phases 4-8) and further clinical examinations (including an OGTT) were performed in 1997-1999 (phase 5 – n=5444/2358) and 2003-2004 (phase 7 – n=4894/2074).(22) The study was approved by the University College London Medical School Committee on the Ethics of Human Research. Informed consent was obtained at baseline and renewed at each contact.

The present case-cohort study is based on a random sample from the source population who attended the phase 3 examination and were followed up to phase 7 (n=8816).(17) We excluded participants with prevalent diabetes at baseline (n=42), missing follow-up data on diabetes (n=552), missing data for key variables (weight, waist circumference, cholesterol, triglycerides, fasting glucose, fasting insulin, and CRP [additionally limited to subjects with CRP <10 mg/l]) at

baseline (n=2018) or during follow-up (phases 5 and 7; n=3049), leading to a case-cohort of 2,810 subjects (335 with incident type 2 diabetes and 2475 without diabetes).

Measurements

Adiponectin

Adiponectin serum concentrations were measured with the Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany). Blood collection, processing and storage followed the same standard operating procedures during all study phases. Venous samples were taken into native tubes in the fasting state (≥5 hours of fasting) before a standard 2-hour OGTT. Samples were centrifuged on site within an hour. Serum was immediately removed from the monovette tubes into microtubes and stored at -80^{o}C. All assays were performed consecutively in the same laboratory (German Diabetes Center), and samples from different study phases of the same participant were measured using the same ELISA plate in order to minimize assay imprecision. Mean intra- and inter-assay CVs were 3.3-5.1% and 12.8-13.8%, respectively. The limit of detection was 3.9 ng/ml. All samples gave values above the limit of detection.

Blood glucose and diabetes

Venous samples for glucose determination were taken into fluoride monovette tubes. Blood glucose was measured using glucose oxidase method.(5) Diabetes was defined by a fasting glucose ≥7.0 mmol/l or a 2h postload glucose ≥11.1 mmol/l using a 75g OGTT.(4) Participants reporting doctor diagnosed diabetes (13.1% of incident cases) or use of glucose-lowering medication (30.4%) were classified as having diabetes regardless of OGTT results. The date of diagnosis

was assigned according to the interval method as the midpoint between the first visit with a diabetes diagnosis and the last visit without diabetes.

Other covariates

The following variables were included as time-invariant covariates: sex, ethnicity (white vs. non-white), early-onset diabetes (<52 years of age at the time of diagnosis – yes/no), and age at the end of follow-up. Body mass index (BMI) and waist circumference (both assessed at each clinical examination contemporaneously with blood draws) were included as time-varying covariates.

Statistical analysis

Statistical analyses were undertaken using SPSS 14.0 statistical software (SPSS Inc., Chicago, IL, USA) and statistical significance was inferred at a 2-tailed $p<0.05$. Due to the skewed distribution of adiponectin values, all analyses use $\log_2$-transformed adiponectin. We compared the characteristics of cases (those who developed type 2 diabetes) and non-cases (those who did not develop diabetes) using t-tests and $\chi 2$-tests as appropriate.

For the subsequent longitudinal analysis, we centred time around the date of diabetes diagnosis for cases and at a randomly selected time point for non-cases to approximate the follow-up time distribution of cases (i.e., $year_0$).(17) Participants were then tracked backwards (retrospectively) to the first clinical screening when adiponectin measurement was obtained (phase 3, the baseline). Of a total of 8233 measurements (964 in cases, 7269 in controls), 2383 measurements were taken after $year_0$ and were excluded from further analysis. The analysis was based on 755 measurements in 335 cases (136 with 3, 148 with 2, and 51 with 1 measurement points) and 5095 measurements in 2474 controls

(743 with 3, 1135 with 2, and 596 with 1 measurement points). As indicated in the table associated with **Figure 1**, adiponectin measurements were well-distributed throughout the 13-year time window of the study.

We used multilevel longitudinal modelling to estimate 13-year adiponectin trajectories before diabetes onset or until $year_0$.(5) Data were structured so that the repeated measurements (person-observations) of adiponectin were nested within subjects and the non-independence of the person-observations was taken into account in estimating standard errors. Differences in adiponectin trajectories between cases and non-cases were modelled using a linear growth model adjusted for age at $year_0$ and ethnicity (both time-invarying covariates).

Non-linearity in adiponectin trajectories was checked by adding quadratic and cubic terms of time-by-caseness interaction to the models; they were all non-significant ($p>0.1$) and thus we describe trajectories only with linear time terms. This is also in agreement with locally-weighted scatterplot smoothers displaying unadjusted associations without making assumptions of the functional form of the association (data not shown).

We investigated the effects of sex and onset of diabetes (early vs. late), first separately and then together, on the trajectories by adding their main effects and time-interactions to the model. Finally we further added the main effect of BMI (and in a separate model waist circumference) to the model as time-varying covariate.

RESULTS

Participants excluded from analysis (n=6006) were generally younger, had a smaller waist circumference, and had higher systolic blood pressure, fasting and

postload blood glucose, were more likely to be female, of lower socioeconomic position, and current smokers at baseline (**Table 1**).

Incident cases (n=335) were older, more obese, had higher blood pressure, fasting and postload glucose, and had lower adiponectin levels than non-cases (n=2475). Cases were less frequently white, more frequently from lower socioeconomic status, and smokers **(Table 2)**.

Adiponectin trajectories by sex

Non-cases had a slight decrease in adiponectin levels over time (-0.34%/year) without significant sex differences (p=0.658). However, males had substantially lower adiponectin levels than women (mean difference: 5358 ng/ml at $year_0$, p<0.0001). (**Figure 1 A, Table 3–Model 1**)

Both male and female diabetes cases had lower adiponectin levels than their same-sex controls throughout follow-up. At diagnosis, the difference was 1131 ng/ml in males and 3181 ng/ml in females (both p<0.0001). While trajectories in cases and controls were parallel in males (p=0.84), female cases had a steeper decline than female controls (slope difference -1.1%/year, p=0.004), resulting in diverging adiponectin trajectories between cases and non-cases over time. (**Figure 1 A, Table 3–Model 1**)

Adiponectin trajectories by age at diabetes onset

Both early-onset (<52 years of age) and late-onset ($\geq$52 years of age) diabetes cases had lower adiponectin levels at diagnosis compared to controls (difference 1907 ng/ml, p=0.001 and 1486 ng/ml, p<0.0001, respectively). In addition, early-onset cases had a steeper decline of adiponectin levels than controls (slope difference: -1.78%/year; p=0.016), while late-onset cases had a parallel slope with

controls (p=0.13 for case-by-time interaction). (**Figure 1 B and C, Table 3–Model 2**)

Adiponectin trajectories by sex and age at onset

The contemporaneous adjustment for sex and age at onset provided similar results. Male and female controls had similar (p=0.66) modest decline in adiponectin over time (-0.34%/year, p<0.0001). Male controls had lower adiponectin levels compared to female controls throughout follow-up (difference at $year_0$: 5355 ng/ml, p<0.0001). (**Figure 1 D and E, Table 3–Model 3**)

All incident diabetes cases had lower adiponectin levels than controls throughout follow-up. At $year_0$, this difference between cases and controls was 1121 ng/ml in late-onset males (p<0.0001), 1295 ng/ml for early-onset males (p=0.005), 3146 ng/ml for late-onset females (p<0.0001), and 3284 ng/ml for early-onset females (p<0.0001) (**Figure 1 D and E, Table 3–Model 3**)

Adiponectin trajectories were parallel for late-onset male cases and controls (p=0.87). In contrast, late-onset female cases showed a steeper decline compared to controls (slope difference: -1.07%/year; p=0.001). (**Figure 1 E, Table 3–Model 3**)

Early-onset cases of both sexes had steeper declines compared to their controls and to the respective late-onset cases (-1.58%/year, p=0.034). (**Figure 1 D, Table 3–Model 3**)

Adjustment for BMI and waist circumference

The downward slope of the adiponectin trajectory in controls was attenuated to non-significance after adjustment for BMI, although the sex difference remained almost the same (5537 ng/ml at $year_0$, p<0.0001). Adjustment for waist

circumference in a separate model changed the slope from a modest decrease to a slight increase (0.38%/year, p<0.0001) in controls and the sex difference was somewhat attenuated (4107 ng/ml at $year_0$, p<0.0001). (**Table 3–Models 4 and 5**)

Adjustment for obesity measures attenuated the difference in adiponectin levels between cases and controls at $year_0$. In early-onset males the attenuation was substantial with adjustment for BMI or waist circumference (from a difference of 18.8% to 10.5% and 11.1%, respectively), neither of the adjusted differences being statistically significant. In late-onset males, the attenuations were also substantial (from 14.7% to 7.3% and 7.3%, respectively), however the differences remained statistically significant. The differences also remained significant both for early- and late-onset females. (**Table 3–Models 4 and 5**)

After adjustment for either BMI or waist, the slope difference between early-onset male cases and controls was attenuated to nonsignificant (p>0.1), while the steeper decline among female cases compared to controls remained almost the same (-0.95%/year for BMI and -0.92%/year for waist adjustment). (**Table 3–Models 4 and 5**)

CONCLUSIONS

In this 13-year longitudinal study of middle-aged British civil servants we found higher adiponectin levels in females compared to males. In people who remained free of diabetes during the study, a modest age-adjusted decrease in adiponectin levels was largely accounted for by increases in obesity. For diabetes cases, the estimated adiponectin levels at diagnosis were lower compared to those in sex-specific controls. This difference was partly explained by obesity. Early-onset diabetes cases of both sexes and late-onset female cases had a steeper decline in pre-diagnosis adiponectin levels compared to sex-specific controls, while parallel

declines were observed for males with late-onset diabetes and controls. The slope difference in early-onset diabetes compared to controls and late-onset cases was largely explained by changes in obesity, whereas the sex-specific slope differences were independent of obesity.

While it is widely accepted that adiponectin is an independent predictor of type 2 diabetes,(14) the actual changes in adiponectin levels are not well-described in humans. A study in spontaneously diabetic Rhesus monkeys described trajectories of obesity and adipokines using several repeat measures,(20) but that study lacked non-diabetic controls. In agreement with our findings, a decreasing linear trajectory in adiponectin was observed in this animal model in relation to the development of obesity and diabetes. Among Rhesus monkeys, the changes in adiponectin and insulin sensitivity were parallel and the observed cross-sectional covariates of adiponectin were insulin sensitivity, weight, fat weight, and plasma insulin. Adiponectin was not related to age or insulin secretion.(20) Our findings suggesting a modest decrease in adiponectin levels with age and that obesity explained part of the cross-sectional differences between cases and controls are in line with the study in monkeys.

Previous human reports on adiponectin changes were based only on 2 time points and thus unable to investigate adiponectin trajectories.(19,21) Reports from population-based cohorts found changes in adiponectin to be inversely associated with weight and postprandial glucose and positively associated with change in HDL cholesterol.(19,21) However, these studies did not investigate the changes separately for diabetes cases and controls. Our finding that the slope differences between early- and late-onset diabetes cases are partially explained by changes in BMI and waist circumference supports an inverse association between changes in obesity and adiponectin levels.

Reports from randomized trials also suggest that changes in adiponectin levels are related to weight changes.(10,18) After 2 years of lifestyle intervention increasing adiponectin levels were found with a decrease in BMI and inflammatory markers.(18) In the Diabetes Prevention Program intensive lifestyle intervention resulted in an increase in adiponectin levels compared to the metformin and placebo groups. Both baseline level and change in weight predicted adiponectin at the end of the follow-up. Higher baseline adiponectin levels (such as those in females) were associated with larger decreases (steeper declines) in adiponectin corresponding to the observations in our study.(10)

The larger separation between incident cases and non-cases among women compared to men during the 13-year follow-up of our study is in agreement with the sex-by-adiponectin interaction observed in some studies investigating the risk of diabetes during a similar or shorter follow-up.(8,11) However, several investigators have not reported (or tested) such interaction effects.(6,9,12,13,15,16) That the slope differences in adiponectin trajectories between early-onset versus late-onset cases and controls were largely explained by changes in obesity means that people with early-onset diabetes are more obese and have a steeper increase in their obesity compared to later-onset cases and controls. Several studies have shown that early-onset diabetes cases are more obese,(24,25) and some have suggested an inverse association between age at onset and BMI at the time of diabetes diagnosis.(26,27)

The finding that adiponectin trajectories preceding diabetes had different slopes in males and females although they were parallel in male and female controls suggests that adiponectin may have different regulation in high-risk male and female subjects. This is unlikely to be related to the adiponectin gene (*ADIPOQ*) that explains ~7% of the phenotypic variation because the gene is

unlikely to cause sex differences in adiponectin levels.(28) A potential explanation could involve sex hormone levels. Adiponectin and sex hormone binding globulin (SHBG) levels have similar bidirectional associations with insulin sensitivity and both are independent predictors of type 2 diabetes. SHBG is involved in sexual dimorphism,(29) and some(30) but not all(31,32) studies have suggested that SHBG is more strongly related to diabetes development in women. Similar sex differences between testosterone levels and type 2 diabetes risk has been reported with higher testosterone levels decreasing the risk in males but increasing in females.(30)

Our results confirm previously observed associations between adiponectin and sex,(33-35) obesity,(6,8,33-38) and diabetes.(14) Due to the low number of non-white participants we were unable to explore the previously described ethnic variation in adiponectin levels(34,35,37) but the potential ethnic differences were controlled for in our analysis. Although some studies report age to be related to adiponectin levels,(33,35,36,39) this association may be confounded by the age-related increase in obesity. Our results show a substantial attenuation in this association after controlling for changes in obesity.

The present study has several limitations. First, some reports suggest that high-molecular-weight adiponectin might be more strongly related to the development of type 2 diabetes(12,14,33) but we were unable to measure adiponectin isoforms in our cohort. Second, it is possible that different adipose tissue compartments are more strongly related to adiponectin levels than BMI or waist circumference.(36,38) Thus, controlling for additional time-varying measures of adiposity might have attenuated the sex differences even further, but we believe that our converging results using 2 measures of obesity validate our findings.

Third, Whitehall II is an occupational cohort, so our findings may not be generalizable to the general population.

Our study benefits from the use of a well-characterized cohort, the use of the gold standard method of diabetes diagnosis, and the up to three times repeated measures of risk factors preceding diabetes diagnosis.(4,22) We applied a sophisticated method to data analysis that account for the interrelationship between within-individual repeated measures. The fact that we were unable to prove any non-linearity of adiponectin development, although we reported nonlinear trajectories of glycemic measures and interleukin-1 receptor antagonist in this dataset, suggests that adiponectin indeed decreases linearly before diabetes diagnosis.(5,17)

In conclusion, we described adiponectin trajectories preceding the diagnosis of type 2 diabetes and compared them with the corresponding trajectories in control participants in a middle-aged cohort of British civil servants. We found significantly lower adiponectin levels in males than females and in participants who developed diabetes compared to those who did not. Adiponectin levels showed a faster decline prior to diabetes in participants with early-onset diabetes and in female cases. While the first was explained by a faster increase in adiposity, the latter may be related to sex-specific mechanisms that relate to both diabetes risk and adiponectin levels.

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Figure legends

Figure 1 – Estimated log-transformed adiponectin trajectories before the diagnosis of diabetes mellitus or end of follow-up in 335 incident diabetes cases and 2475 controls.

(A) Adiponectin trajectories by sex and incident diabetes status

(B) Adiponectin trajectories by incident diabetes status in cases with early-onset diabetes

(C) Adiponectin trajectories by incident diabetes status in cases with late-onset diabetes

(D) Adiponectin trajectories by sex and incident diabetes status in cases with early-onset diabetes

(E) Adiponectin trajectories by sex and incident diabetes status in cases with late-onset diabetes

Multilevel longitudinal modeling using linear growth models. All models are adjusted for age at the end of follow-up and ethnicity (white/non-white), (A) shows a model additionally adjusted for sex, (B) and (C) is additionally adjusted for age at onset (early-onset - 52 years of age at diagnosis), and (D) and (E) are adjusted for both sex and age at onset. Estimated for a hypothetical population of 72% male, 92% white, aged 63 years (A, C, E) or 50 years (B, D) at $year_0$. Error bars show 95% confidence intervals for the fixed effects. Tables show the number of measurements for each year at and before diabetes diagnosis / end of follow-up.

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Tables

Table 1 – Characteristics of participants excluded and included in the current analysis at study baseline.

	Non-participants	Participants	P
N	6006	2810	
Age (years)	50.7±6.1	49.3±5.9	<0.0001
BMI (kg/m^2)	25.3±3.8	25.3±3.5	0.328
Waist circumference (cm)	83.6±11.7	84.2±11.2	0.041
Systolic blood pressure (mmHg)	121±14	120±10	0.001
Diastolic blood pressure (mmHg)	80±10	80±9	0.525
Fasting blood glucose (mmol/L)	5.3±0.8	5.2±0.5	0.001
2-hour blood glucose (mmol/L)	5.7±2.2	5.5±1.5	<0.0001
Male	4011 (66.8)	2047 (72.8)	<0.0001
White	5386 (90.3)	2659 (91.5)	0.084
Social grade			<0.0001
Administrative	2020 (36.7)	1152 (41.1)	
Executive	2440 (44.3)	1303 (46.5)	
Support	1049 (19.0)	348 (12.4)	
Smoker	827 (15.0)	318 (11.3)	<0.0001

Data are mean±SD, median [interquartile range], or n (%).

Table 2 – Baseline characteristics of incident diabetes cases and non-cases.

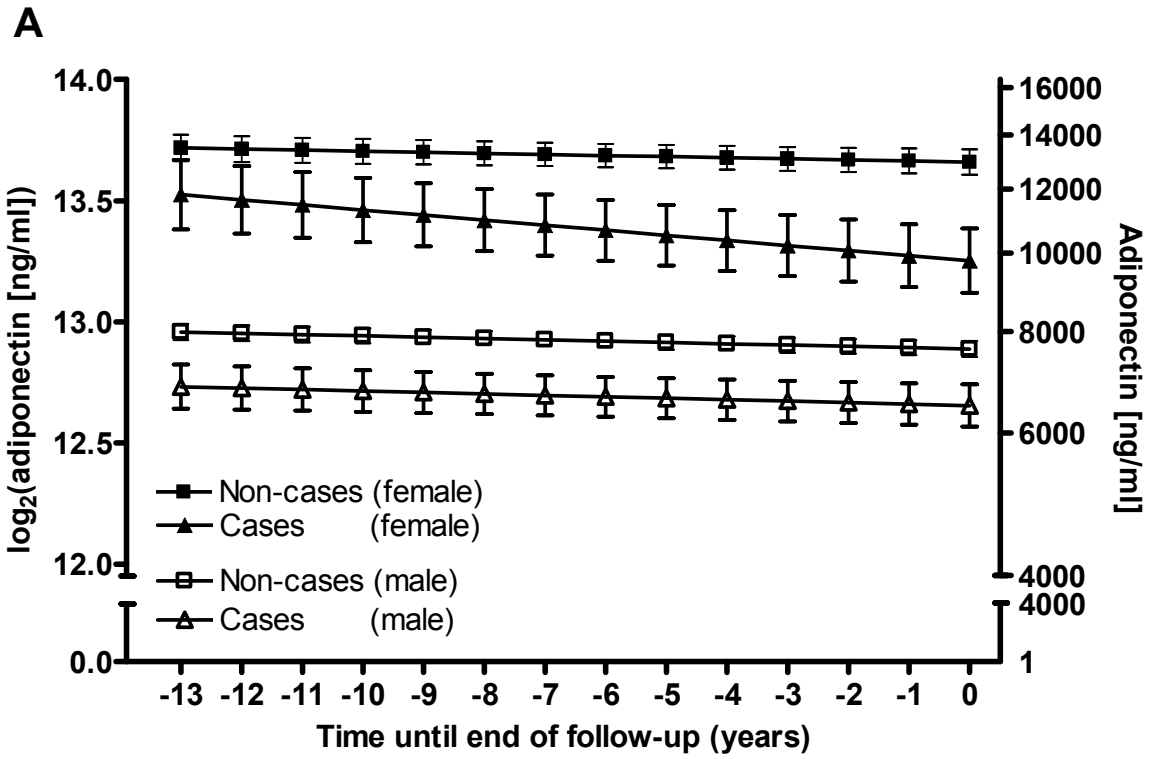
	Non-cases	Cases	P
N	2475	335	
Age (years)	49.1±5.8	51.0±6.1	<0.0001
BMI (kg/m^2)	25.0±3.3	27.1±4.2	<0.0001
Waist circumference (cm)	83.5±10.8	89.2±12.2	<0.0001
Systolic blood pressure (mmHg)	119±13	124±14	<0.0001
Diastolic blood pressure (mmHg)	79±9	82±10	<0.0001
Fasting blood glucose (mmol/L)	5.2±0.4	5.5±0.5	<0.0001
2-hour blood glucose (mmol/L)	5.3±1.4	6.7±1.9	<0.0001
Male	1813 (73.3)	234 (69.9)	0.19
White	2299 (92.9)	270 (80.6)	<0.0001
Social grade			<0.0001
Administrative	1066 (43.1)	86 (25.9)	
Executive	1127 (45.6)	176 (53.0)	
Support	278 (11.3)	70 (21.1)	
Smoker	262 (10.6)	56 (16.9)	0.001
Early-onset (<52 yrs of age) diabetes	NA	52 (15.5)	
Adiponectin (ng/ml)	8817[6534-12,369]	7140[5187-10,304]	<0.0001

Data are mean±SD, median [interquartile range], or n (%)

Table 3 – Fixed effects for multilevel models of change over time of log_2(adiponectin) concentrations before diabetes diagnosis or end of follow-up.

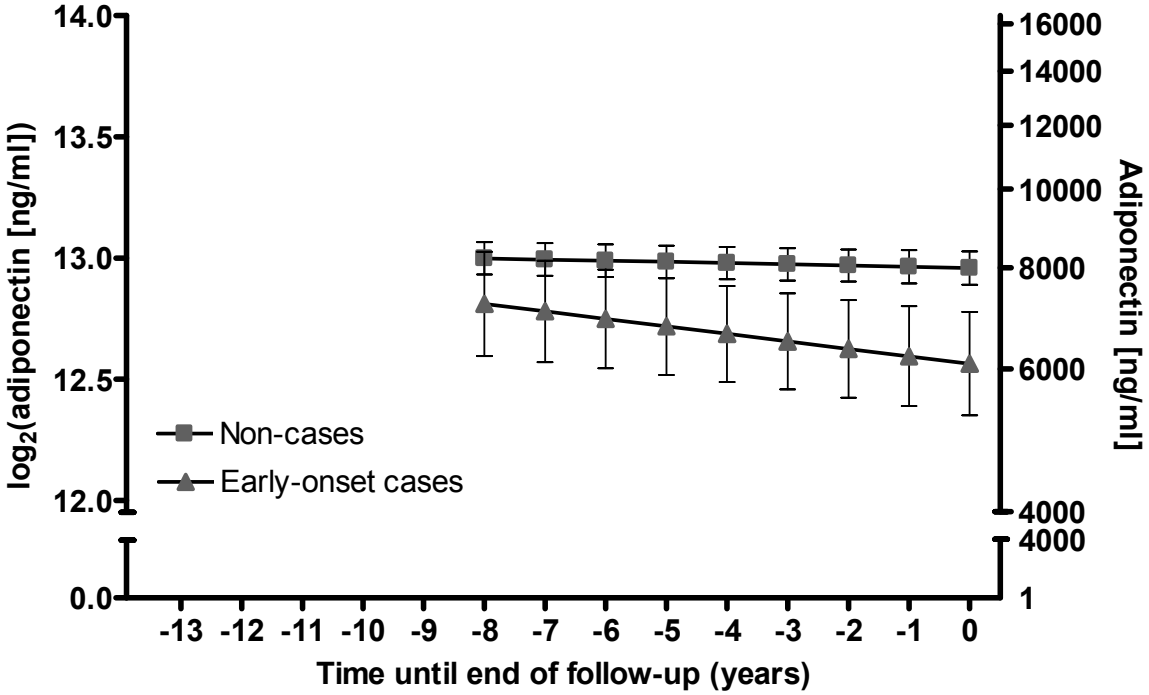
	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**
Incident diabetes	-0.23(0.05)*	NA	NA	NA	NA
Early-onset diabetes	NA	-0.40(0.12)†	-0.30(0.11)‡	NS	NS
Late-onset diabetes	NA	-0.27(0.05)*	-0.23(0.05)*	-0.11(0.05)§	-0.11(0.05)§
Female	0.77(0.03)*	NA	0.77(0.03)*	0.78(0.03)*	0.59(0.03)*
Diabetes x Female	-0.17(0.09)§	NA	-0.17(0.09)§	-0.17(0.08)§	-0.17(0.08)§
Time (per year)	-0.005(0.001)*	-0.012(0.003)*	-0.005(0.001)*	NS	0.006(0.001)*
Female x Time	NS	NA	NS	NS	NS
Diabetes x Time	NS	NS	NS	NS	NS
Early-onset diabetes x Time	NA	-0.026(0.011)§	-0.023(0.011)§	NS	NS
Diabetes x Female x Time	-0.016 (0.006)‡	NA	-0.016(0.005)†	-0.014(0.005)‡	-0.013(0.005)‡
BMI (per 1 kg/m2)	NA	NA	NA	-0.048(0.002)*	NA
Waist (per cm)	NA	NA	NA	NA	-0.016(0.001)*

Data are regression coefficients (SE). All models were adjusted for age at the end of follow-up and ethnicity (white/non-white). Model 1 is also adjusted for sex, model 2 for early-onset diabetes (<52 years of age at diabetes diagnosis), model 3 for both sex and early-onset diabetes, model 4 is model 3 further adjusted for BMI, model 5 is model 3 further adjusted for waist circumference. Trajectories in 335 incident diabetes cases and 2475 controls. Models with the lowest information criteria are shown. * P<0.0001, † P<0.001, ‡ P<0.01, § P<0.05. NA, not applicable (variable not included in the model); NS, not significant (P>0.05), term dropped from the final model.

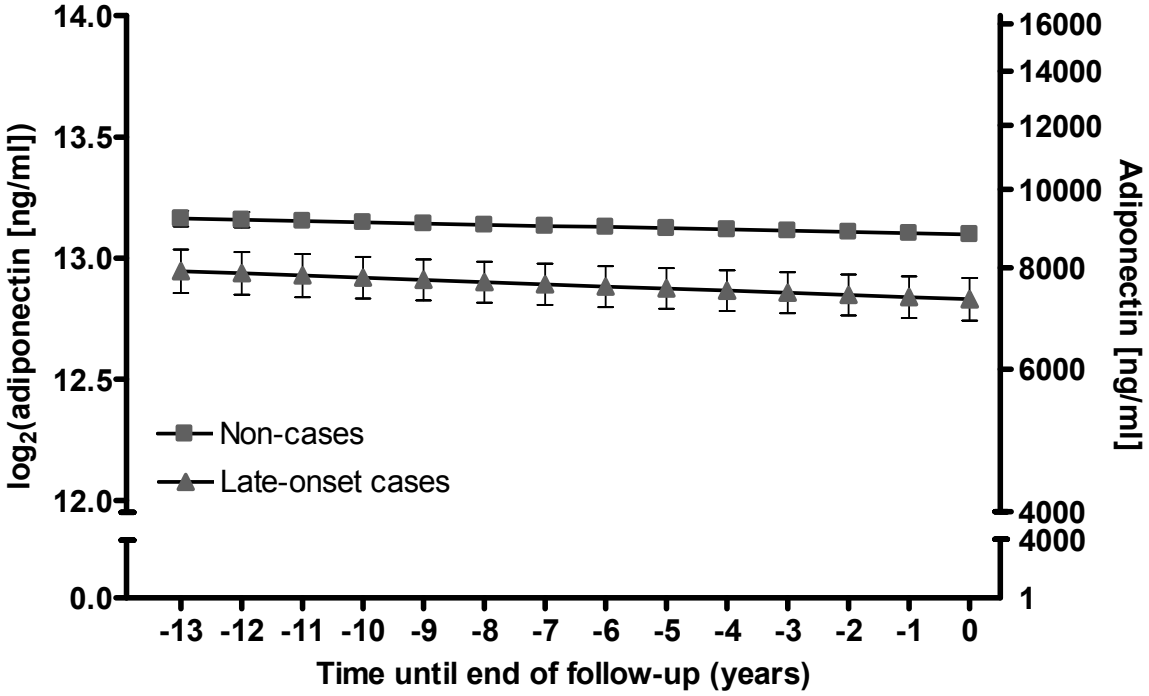


Controls	214	474	476	239	53	104	409	805	682	236	63	308	654	378
Incident diabetes	38	46	73	30	9	11	44	112	82	36	6	49	36	183

B


14.0
13.5
13.0
12.5
12.0
0.0
log2(adiponectin [ng/ml])
16000
14000
12000
10000
8000
6000
4000
4000
1
Adiponectin [ng/ml]
Non-cases
Early-onset cases
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
Time until end of follow-up (years)

C


14.0
13.5
13.0
12.5
12.0
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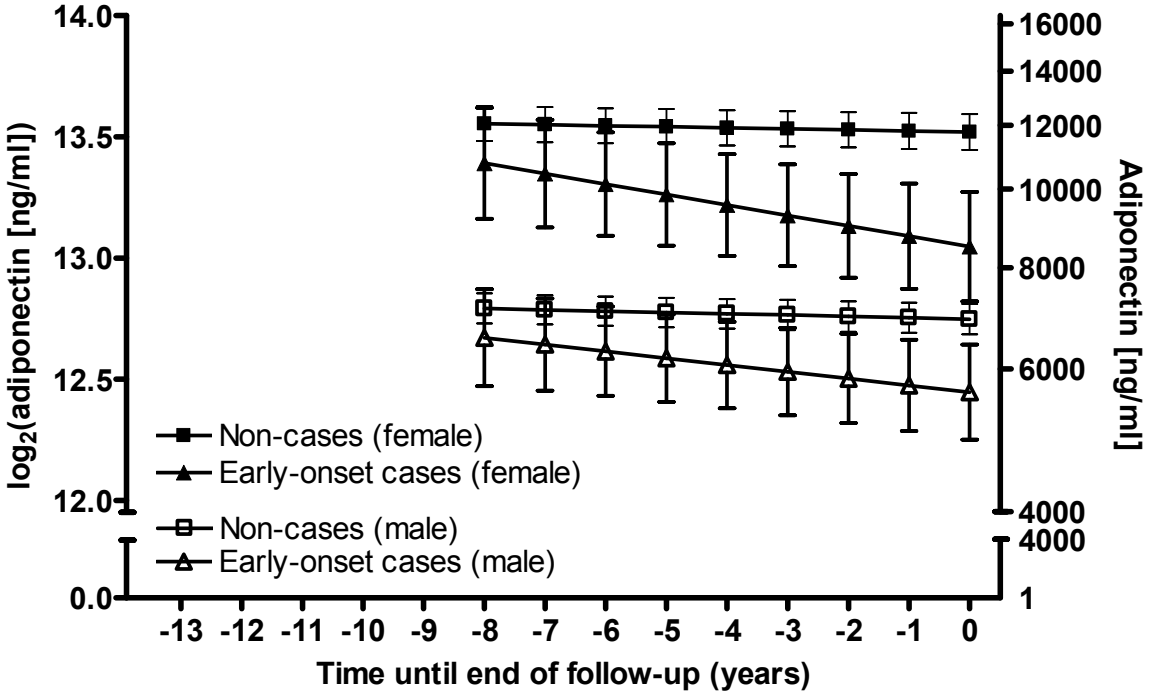
log2(adiponectin [ng/ml])
16000
14000
12000
10000
8000
6000
4000
4000
1
Adiponectin [ng/ml]
Non-cases
Late-onset cases
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
Time until end of follow-up (years)

D


14.0
13.5
13.0
12.5
12.0
0.0
log2(adiponectin [ng/ml])
16000
14000
12000
10000
8000
6000
4000
4000
1
Adiponectin [ng/ml]
Non-cases (female)
Early-onset cases (female)
Non-cases (male)
Early-onset cases (male)
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
Time until end of follow-up (years)

E

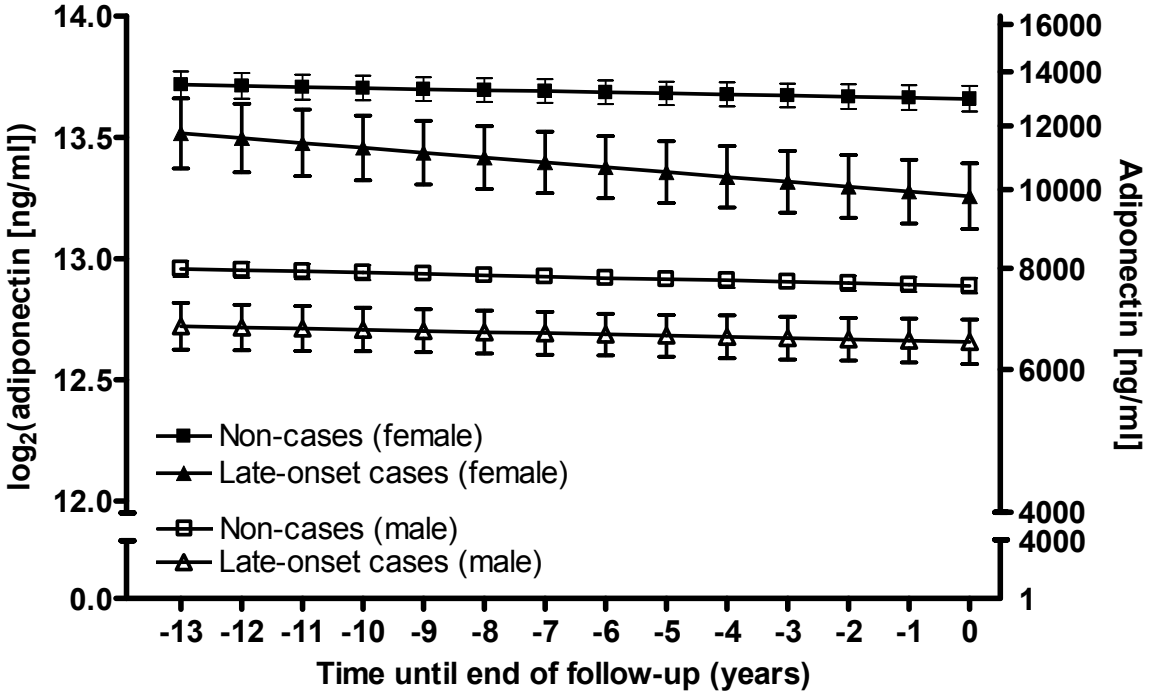

14.0
13.5
13.0
12.5
12.0
0.0
log2(adiponectin [ng/ml])
16000
14000
12000
10000
8000
6000
4000
4000
1
Adiponectin [ng/ml]
Non-cases (female)
Late-onset cases (female)
Non-cases (male)
Late-onset cases (male)
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
Time until end of follow-up (years)

Figure 1 – Estimated log-transformed adiponectin trajectories before the diagnosis of diabetes mellitus or end of follow-up in 335 incident diabetes cases and 2475 controls.

(A) Adiponectin trajectories by sex and incident diabetes status

(B) Adiponectin trajectories by incident diabetes status in cases with early-onset diabetes

(C) Adiponectin trajectories by incident diabetes status in cases with late-onset diabetes

(D) Adiponectin trajectories by sex and incident diabetes status in cases with early-onset diabetes

(E) Adiponectin trajectories by sex and incident diabetes status in cases with late-onset diabetes

Multilevel longitudinal modeling using linear growth models. All models are adjusted for age at the end of follow-up and ethnicity (white/non-white), (A) shows a model additionally adjusted for sex, (B) and (C) is additionally adjusted for age at onset (early-onset - 52 years of age at diagnosis), and (D) and (E) are adjusted for both sex and age at onset. Estimated for a hypothetical population of 72% male, 92% white, aged 63 years (A, C, E) or 50 years (B, D) at $year_0$. Error bars show 95% confidence intervals for the fixed effects. Tables show the number of measurements for each year at and before diabetes diagnosis / end of follow-up.

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Author: 1st authorship shared with Dr. Adam G. Tabák

Chapter 4

Macrophage Inhibitory Cytokine-1 (MIC-1) is Increased in Individuals Before Type 2 Diabetes Diagnosis but is not an Independent Predictor of Type 2 Diabetes: The Whitehall II Study

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CLINICAL STUDY

Macrophage inhibitory cytokine-1 is increased in individuals before type 2 diabetes diagnosis but is not an independent predictor of type 2 diabetes: the Whitehall II study

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Abstract

Objective: Macrophage inhibitory cytokine-1 (MIC-1) belongs to the transforming growth factor (TGF)-β superfamily, and has been reported to be involved in energy homoeostasis and weight loss and to have anti-inflammatory properties. We hypothesized that decreased concentrations of MIC-1 would be associated with higher risk of developing type 2 diabetes.

Design and methods: We designed a nested case–control study within the Whitehall II cohort and measured serum concentrations of MIC-1 by ELISA in 180 individuals without type 2 diabetes at baseline who developed type 2 diabetes during the follow-up period of 11.5 ± 3.0 years and in 372 controls frequency-matched for age, sex, and body mass index with normal glucose tolerance throughout the study.

Results: MIC-1 concentrations at baseline were higher in cases (median (25/75th percentiles) 537.1 (452.7–677.4) pg/ml) than in controls (499.7 (413.8–615.4) pg/ml; $P=0.0044$). In the age- and sex-adjusted model, a 1-SD. increase in MIC-1 (206.0 pg/ml) was associated with an odds ratio (95% confidence interval) of 1.21 (0.997; 1.46; $P=0.054$) for type 2 diabetes. Adjustment for waist circumference, cardiovascular risk factors, socioeconomic status, proinflammatory mediators, and glycemia abolished the association.

Conclusions: Baseline MIC-1 concentrations were increased, not decreased, in individuals before type 2 diabetes manifestation, but not independently associated with incident type 2 diabetes in multivariable analyses. This upregulation of MIC-1 could be part of an anti-inflammatory response preceding the onset of type 2 diabetes, which has been described before for interleukin-1 receptor antagonist and TGF-β1.

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Introduction

Serum levels of several pro- and anti-inflammatory cytokines are elevated in individuals who will subsequently develop type 2 diabetes (1, 2), and so far adiponectin is the only adipokine for which an inverse association of serum levels with risk for type 2 diabetes has been described (3). Macrophage inhibitory cytokine-1 (MIC-1), a member of the transforming growth factor (TGF)-β superfamily (4), has emerged as a candidate anti-inflammatory link due to its regulatory role in energy homoeostasis with elevated levels being associated with weight loss (5). MIC-1 is expressed in several tissues throughout the body including liver, kidney, and adipose tissue (6). Although MIC-1 is expressed in adipose tissue and secreted from adipocytes, an inverse correlation between *MIC-1* gene expression and measures of obesity has been reported (6). Basic physiological research also points to an inverse association between proinflammatory cytokines and MIC-1. *MIC-1* gene expression is downregulated by leptin and interleukin (IL)-1β, whereas MIC-1 increases the release of adiponectin from adipocytes (6), and attenuates lipopolysaccharide-induced tumor necrosis factor-α release from macrophages (4). Macrophages have been implicated in different aspects of diabetes development. Several studies reported an increased number of macrophages in pancreatic islets from patients with type 2 diabetes (7) and in adipose tissue of obese individuals (8–10) as well as higher activation

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of peripheral blood monocytes in patients with poorly controlled diabetes (11). Activated macrophages secrete proinflammatory and chemotactic cytokines and chemokines that can impair β-cell function and adipocyte insulin sensitivity, and stimulate further activation and infiltration of monocytes into these tissues (1).

Data from prospective studies on MIC-1 and risk for type 2 diabetes are not yet available. A small cross-sectional study recently reported elevated, not decreased, serum levels of MIC-1 in obesity and type 2 diabetes (12). However, based on the aforementioned reports that pointed toward anti-inflammatory properties of MIC-1, we hypothesized that MIC-1 could be regulated in a similar manner as adiponectin, and that increased serum concentrations would be associated with decreased risk for type 2 diabetes. The aims of this study were to examine the relationship of MIC-1 serum concentration with anthropometric, metabolic, and inflammatory variables and to characterize its association with incident type 2 diabetes.

Methods

Study design

The presented data are from a nested case–control study within the Whitehall II cohort. The Whitehall II study started in 1985 and included 10 308 civil servants aged 35–55 years in phase 1 (1985–1988). A detailed description of the cohort profile was given previously (13).

The baseline for the current study was phase 3 ($n=7537$) (1991–1994) as this was the first phase including a 75 g oral glucose tolerance test (OGTT). In phases 4–8, participants were followed through postal questionnaires at ~2.5-year intervals. Clinical examinations including an OGTT were additionally performed at phases 3, 5, and 7.

All participants selected for the current nested case–control study ($n=552$) were diabetes free at baseline (phase 3). Individuals who developed type 2 diabetes during the follow-up period of 11.5 ± 3.0 years served as cases ($n=180$). Controls ($n=372$) with normal glucose tolerance at baseline and during follow-up were frequency-matched to cases for age (5-year bands), sex, and body mass index (BMI; 5 kg/m^2 bands). The study population is slightly smaller than the sample from a previous report (2) as no serum for MIC-1 assays was available for seven study participants.

Individuals with prevalent or incident cardiovascular disease, incident coronary heart disease, self-reported longstanding inflammatory illness, recent inflammatory symptoms, anti-inflammatory medication, and/or non-white ethnicity were excluded.

Experimental and statistical analyses

Serum concentrations of MIC-1 (growth differentiation factor-15, GDF-15) were measured with the Quantikine Human GDF-15 Immunoassay kit (R&D Systems, Wiesbaden, Germany). Mean intra-assay and inter-assay coefficients of variation were 2.7 and 3.3% respectively. The limit of detection was 23.4 pg/ml. All samples gave values above the limit of detection.

Univariate associations of MIC-1 with anthropometric, metabolic, and immunological variables were estimated using linear regression. Logistic regression was used to assess the association between MIC-1 levels, possible covariates and incident type 2 diabetes. Results are given as odds ratios (OR) and corresponding 95% confidence intervals (95% CI). For all statistical analyses, $P<0.05$ was considered to be statistically significant. All statistical analyses were performed with the statistical computer software SAS (version 9.2, SAS Institute, Cary, NC, USA).

Results

Study population

Characteristics of an almost identical study population have been described before (2), and baseline characteristics of participants stratified in controls ($n=372$) and cases ($n=180$) are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). Cases had higher waist circumference (men only), blood pressure, serum levels of fasting triglycerides, fasting and 2-h glucose as well as fasting insulin, higher serum concentrations of MIC-1, IL-1Ra and C-reactive protein (CRP) compared with the controls. Moreover, cases had lower insulin sensitivity, and were more frequently on antihypertensive medication.

Cross-sectional associations of MIC-1 with anthropometric, metabolic, and immunological variables

Age showed a strong positive association with serum MIC-1 concentrations, and weaker associations were present with BMI, waist circumference, blood pressure, total cholesterol, and fasting triglycerides (Table 1). Fasting insulin and homeostasis model assessment of insulin resistance were positively associated with MIC-1, whereas neither fasting nor 2-h blood glucose was associated with MIC-1. Regarding pro- and anti-inflammatory immune mediators, CRP, IL-6, and IL-1Ra, but not adiponectin, showed a positive association with MIC-1. In subanalyses, associations tended to be stronger among cases than controls.

Table 1 Association between baseline serum macrophage inhibitory cytokine-1 (log_2 transformed) and anthropometric, metabolic, and immunological variables.

Variables	**Controls** ($n=372$) β (95% CI)	**Cases** ($n=180$) β (95% CI)	**All** ($n=552$) β (95% CI)
Anthropometric factors			
Age (years)	0.025 (0.018; 0.032)‡	0.034 (0.022; 0.045)‡	0.028 (0.022; 0.034)‡
BMI (kg/m^2)	0.008 (−0.004; 0.020)	0.024 (0.008; 0.039)†	0.016 (0.007; 0.026)‡
Waist circumference (cm)	0.003 (−0.001; 0.007)	0.011 (0.005; 0.016)‡	0.006 (0.003; 0.010)‡
Systolic blood pressure (mmHg)	0.003 (−0.001; 0.006)	0.008 (0.004; 0.013)‡	0.005 (0.003; 0.008)‡
Diastolic blood pressure (mmHg)	0.004 (−0.001; 0.009)	0.009 (0.002; 0.015)*	0.006 (0.002; 0.010)†
Metabolic factors			
Total cholesterol (mmol/l)	0.043 (0.004; 0.081)*	0.063 (0.001; 0.124)*	0.054 (0.021; 0.086)†
Fasting triglycerides (mmol/l)	0.090 (0.035; 0.145)†	0.128 (0.042; 0.215)†	0.113 (0.067; 0.159)‡
Fasting glucose (mmol/l)	0.068 (−0.060; 0.196)	0.015 (−0.125; 0.154)	0.061 (−0.025; 0.147)
2-h glucose (mmol/l)	0.005 (−0.037; 0.046)	0.011 (−0.029; 0.052)	0.023 (−0.002; 0.048)
Fasting insulin (mU/l)	0.047 (−0.005; 0.099)	0.126 (0.046; 0.205)†	0.084 (0.042; 0.126)‡
HOMA-IR	0.048 (−0.003; 0.099)	0.119 (0.042; 0.195)†	0.081 (0.041; 0.121)‡
Immunological factors			
CRP (mg/l)	0.071 (0.041; 0.102)‡	0.068 (0.026; 0.110)†	0.075 (0.051; 0.100)‡
IL-6 (pg/ml)	0.121 (0.060; 0.181)‡	0.149 (0.049; 0.249)†	0.135 (0.082; 0.187)‡
IL-1Ra (pg/ml)	0.096 (0.020; 0.172)*	0.157 (0.059; 0.256)†	0.141 (0.083; 0.200)‡
Adiponectin (ng/ml)[a]	−0.0026 (−0.107; 0.102)	−0.096 (−0.216; 0.024)	−0.051 (−0.129; 0.026)

Data are from linear regression analysis, and are given as β values, 95% CI, and corresponding P values. Triglycerides, insulin, HOMA-IR, C-reactive protein (CRP), interleukin (IL)-6, IL-1Ra and adiponectin entered the models as log_2-transformed variables. *$P<0.05$; †$P<0.01$; ‡$P<0.001$.
[a]Adiponectin concentrations were available for a subset of study participants (158 controls and 139 cases).

MIC-1 and incident diabetes

Table 2 shows the association between circulating concentrations of MIC-1 and incident diabetes. MIC-1 concentrations at baseline were higher in cases (median 537.1 pg/ml (25–75th percentiles 452.7–677.4)) than in controls (499.7 (413.8–615.4); $P=0.0044$). In the age- and sex-adjusted model, the association between a 1-S.D. increase of MIC-1 (206.0 pg/ml) and increased risk for type 2 diabetes was borderline significant (OR (95% CI) 1.21 (0.997; 1.46)). Additional adjustment for waist circumference, cardiovascular risk factors, socioeconomic status, proinflammatory mediators, or glycemia abolished the association.

Discussion

Our nested case–control study within the Whitehall II study is the first prospective investigation of the association between MIC-1 serum concentrations and incident type 2 diabetes. We found that MIC-1 levels were significantly higher in individuals who subsequently developed type 2 diabetes than those who remained diabetes free. However, there was only a borderline significant association between MIC-1 and incident type 2 diabetes after adjustment for age and sex, which was further attenuated after adjustment for waist circumference, cardiovascular risk factors, socioeconomic status, proinflammatory mediators,

Table 2 Association between circulating concentrations of macrophage inhibitory cytokine-1 at baseline and incident type 2 diabetes.

Model	Covariables	**OR** (95% CI)	*P*
1	Age, sex	1.21 (0.997; 1.46)	0.054
2	Age, sex, and waist circumference	1.15 (0.95; 1.40)	0.16
3	Model 2+ cardiovascular risk factors (cholesterol, fasting triglycerides, blood pressure, smoking[a], physical activity[b], antihypertensive medication, and lipid-lowering medication)	1.06 (0.86; 1.30)	0.58
4	Model 2+ socioeconomic status (employment grade[c])	1.11 (0.91; 1.35)	0.29
5	Model 2+ proinflammatory mediators (CRP; IL-6)	1.12 (0.92; 1.38)	0.26
6	Model 2+ glycemia (fasting glucose, 2-h glucose and fasting insulin)	1.12 (0.88; 1.42)	0.35
7	Combination of all covariables from models 2–6	1.05 (0.78; 1.41)	0.75

Data are given as odds ratio (95% CI) for a 1-S.D. increase (206.0 pg/ml) of MIC-1 concentrations. Triglycerides, C-reactive protein (CRP), interleukin (IL)-6, and insulin entered the models as ln-transformed variables.
[a]Smoking is coded in three classes (never smoked, former smoker, and current smoker).
[b]Physical activity is coded in three classes (none/mild, moderate, and vigorous).
[c]Employment grade is coded in six classes running from 1 (highest grade) to 6 (lowest grade).

and glycemia. This reduction in the effect size is in line with the positive associations we observed between MIC-1 and potential confounders which are known risk factors for type 2 diabetes.

Our main finding does not support the hypothesis that MIC-1 could be, through its anti-inflammatory effects, a protective cytokine against the development of type 2 diabetes. Our data extend current knowledge on the role of MIC-1 in the development of type 2 diabetes. So far, there is only one report from a small cross-sectional study including 54 women that reported increased concentrations of MIC-1 in patients with type 2 diabetes (12). Due to its cross-sectional design, it could not exclude the possibility that elevated concentrations of MIC-1 were a consequence and not a cause of type 2 diabetes. We demonstrate that MIC-1 is already increased over 11 years before the manifestation of type 2 diabetes.

MIC-1 may be a part of a counterregulatory, anti-inflammatory response which is independently regulated from adiponectin. We used the same study sample before to show that serum levels of the anti-inflammatory cytokine IL-1Ra were elevated before the diagnosis of type 2 diabetes (2). The concept of the presence of an anti-inflammatory counterregulation before type 2 diabetes is further supported by data from the MONICA/KORA study that demonstrates an association between elevated serum levels of the anti-inflammatory and immunosuppressive cytokine TGF-β1 and increased risk of type 2 diabetes (14). Taken together, these findings regarding type 2 diabetes should be seen in context with the general regulatory principle in inflammation that in acute conditions, proinflammatory stimuli at the beginning of the process induce anti-inflammatory mediators in order to resolve inflammation (15). In individuals with an increased risk for type 2 diabetes, a chronic state of immune activation can be found. The concomitant and persistent upregulation of both pro-and anti-inflammatory cytokines in these individuals may reflect their inability to efficiently counterregulate inflammatory stimuli (16).

Our data indicate that despite higher levels in cases than in controls in the unadjusted analysis, MIC-1 by itself is not associated with the risk for type 2 diabetes independently of established risk factors. It is interesting to note that circulating levels of MIC-1 have been found to be independent predictors of cardiovascular events and mortality (17–19). There are some putative explanations why our findings were contrary to our hypothesis. In the study by Johnen *et al.* (5), the association between higher MIC-1 and lower BMI was consistent across several mouse models, whereas associations with low BMI and weight loss in man were detected in patient samples with prostate cancer or chronic renal failure which clearly differ from our study population of initially healthy middle-aged men and women. Moreover, we did not confirm the previously observed inverse correlation between *MIC-1* gene expression and BMI (6). However, this finding was based on measurements with MIC-1 mRNA in adipose tissue from small study samples with a very wide BMI range ($\sim$20–80 kg/m^2), whereas our data represent a much lower average BMI. In addition, we measured MIC-1 protein concentrations in the circulation which we believe to be an integrated measure of MIC-1 secretion and turnover in many body tissues.

Our study has several limitations and strengths. Point estimates and CIs are derived from non-weighted data from a nested case–control study and therefore, statistical inferences based upon them may be restricted and may not represent the best available estimate within the context of the original cohort. Furthermore, the cohort consists of employees of the British Civil Service who were mainly male and middle-aged. However, this is, to the best of our knowledge, the first prospective study to analyze whether MIC-1 serum levels were associated with incident type 2 diabetes. In addition, our analyses are based on a well-phenotyped cohort.

In conclusion, we found that increased MIC-1 levels were not independently associated with incident type 2 diabetes, although MIC-1 levels were elevated in individuals before type 2 diabetes manifestation in the unadjusted analysis.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-09-1066.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Supplementary Table 1 Characteristics of study participants stratified by case-control status

Variables	**Controls (*N* = 372)**	**Cases (*N* = 180)**	***P***
Age (years)	50.6 ± 6.3	51.1 ± 5.9	0.43
Sex (m/f) (%)	73/27	72/28	0.76
BMI (kg/m²)	26.3 ± 3.8	27.2 ± 4.5	0.055
Waist circumference (men) (cm)	89.8 + 9.6	92.3 + 10.4	0.022
Waist circumference (women) (cm)	78.2 + 11.5	83.8 + 16.0	0.057
Systolic blood pressure (mmHg)	121.1 ± 12.6	125.9 ± 15.0	0.0003
Diastolic blood pressure (mmHg)	80.6 ± 9.3	83.0 ± 10.6	0.0036
Total cholesterol (mmol/l)	6.6 ± 1.2	6.7 ± 1.2	0.34
Fasting triglycerides (mmol/l)	1.2 (0.9; 1.8)	1.6 (1.2; 2.4)	< 0.0001
Fasting glucose (mmol/l)	5.1 ± 0.4	5.4 ± 0.6	< 0.0001
2h glucose (mmol/l)	5.1 ± 1.2	6.6 ± 1.9	< 0.0001
Fasting insulin (mU/l)	5.2 (3.3; 7.8)	7.9 (5.4; 12.0)	< 0.0001
HOMA-IR	1.2 (0.7; 1.8)	1.9 (1.3; 3.0)	< 0.0001
MIC-1 (pg/ml)	499.7 (413.8; 615.4)	537.2 (452.7; 677.4)	0.0044
IL-1Ra (pg/ml)	207.4 (158.4; 274.8)	233.5 (180.4; 343.2)	0.0004
CRP (mg/l)	0.9 (0.5; 1.6)	1.1 (0.6; 2.4)	0.0058
IL-6 (pg/ml)	1.4 (1.1; 2.0)	1.6 (1.2; 2.1)	0.086
Employment grade [a] (%)	18.4/23.9/14.8/ 16.5/13.5/12.9	16.2/18.1/15.7/ 16.9/10.2/22.9	0.071
Smoking status [b] (%)	53.4/35.5/11.1	51.2/37.8/11.0	0.88
Physical activity [c] (%)	33.5/50.0/16.5	37.1/45.5/17.5	0.62
Antihypertensive medication (yes/no) (%)	6.5/93.5	11.7/88.3	0.036
Lipid-lowering medication (yes/no) (%)	0.3/99.7	1.1/98.9	0.21

Data with normal and non-normal distribution are given as mean ± standard deviation and median (25^{th} percentile; 75^{th} percentile), respectively. Groups were compared using chi-square test, Fisher's exact test, two-sample t-test and Wilcoxon test as appropriate.

[a] Employment grade is coded in 6 classes running from 1 (highest grade) to 6 (lowest grade);

[b] Smoking is coded in 3 classes (never smoked, former smoker, current smoker);

[c] Physical activity is coded in 3 classes (none/mild, moderate, vigorous).

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Conceived / designed experiments:	50%
Performed experiments:	100%
Analysed data:	30%
Contributed to discussion:	50%
Wrote the manuscript:	70%
Reviewed / edited manuscript:	30%

Author: 1st author

Chapter 5

Relationship of Serum Concentrations of the Novel Antagonistic Adipokines Sfrp5 and Wnt5a with Clinical and Metabolic Variables in Humans

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Relationship of serum concentrations of the novel antagonistic adipokines Sfrp5 and Wnt5a with clinical and metabolic variables in humans

Short title: Sfrp5, Wnt5a and clinical/metabolic variables

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Conflict of interest

The authors declare no conflict of interest.

Abstract

Objective:

Secreted frizzled-related protein 5 (Sfrp5) has recently been described as novel adipokine in mice with insulin-sensitising and anti-inflammatory properties similar to adiponectin. The aim of this study was to measure serum concentrations and determinants of Sfrp5 and its proinflammatory antagonist Wnt5a and to compare them with adiponectin in humans.

Subjects and methods:

Serum concentrations of Sfrp5, Wnt5a and adiponectin were measured in 47 study participants who participated in a coffee intervention study. Associations with demographic, metabolic and immunological variables and regulation of serum levels by different amounts of daily coffee intake were analysed.

Results:

At baseline fasting serum Sfrp5 levels ranged between 96 and 2 395 ng/ml. Serum Wnt5a concentrations were below the detection limit (0.02 ng/ml) in 80.9% of the study participants. Sfrp5 was directly correlated insulin resistance (measured as homeostasis model assessment-insulin resistance/HOMA-IR; $r=0.32$, $p<0.05$) and with the oxidative stress markers 8-isoprostane ($r=0.44$, $p<0.01$) and nitrotyrosine ($r=0.52$, $p<0.001$), whereas adiponectin showed inverse correlations with several indices of insulin resistance (e.g. HOMA-IR, Stumvoll index; all $p<0.05$) and a direct correlation with the antiatherogenic apolipoprotein A-I ($r=0.56$, $p<0.001$). Serum Wnt5a and the ratio Sfrp5/Wnt5a were directly associated with markers of subclinical inflammation, macrophage migration inhibitory factor ($r=0.30$, $p<0.05$) and interleukin-18 ($r=0.39$, $p<0.01$), respectively. None of the measured adipokines were associated with demographic and anthropometic variables.

Coffee did not affect serum Sfrp5, whereas adiponectin was upregulated and Wnt5a was downregulated.

Conclusion:

In contrast to obese mouse models, serum Sfrp5 was directly correlated with HOMA-IR and oxidative stress, but not with apolipoproteins and subclinical inflammation and thus differed from associations found for circulating adiponectin and Wnt5a. Sfrp5 was also not upregulated by coffee like adiponectin, whereas Wnt5a was downregulated. These data point towards species-specific differences in metabolic and immunological correlates and in regulatory mechanisms between Sfrp5 and adiponectin.

Introduction

Obesity is a major risk factor of cardiometabolic diseases such as type 2 diabetes and atherosclerosis, which are all characterised by low-grade inflammation and insulin resistance (1-4). In obesity, expression and release of several adipokines with immunomodulatory characteristics is altered in adipose tissue, and their secretion is strongly correlated with adipocyte size (5). Most of the proinflammatory cytokines and chemokines such as interleukin (IL)-6, IL-8, IL-18, monocyte chemoattractant protein (MCP)-1, macrophage migration inhibitory factor (MIF) and leptin are released at higher levels in obesity (5-8), whereas the anti-inflammatory adipokine adiponectin is released in lower amounts (9).

Secreted frizzled-related protein 5 (Sfrp5) was recently identified as novel adipokine with anti-inflammatory properties (10). It belongs to the Sfrp family and antagonises Wnt5a. Both proteins are expressed in adipose tissue and involved in the non-canonical Wnt signaling. In obese and diabetic mice, adipose tissue Sfrp5 levels were reduced, whereas Wnt5a levels in adipose tissue were increased,

resulting in a decrease of the ratio of Sfrp5/Wnt5a. Genetic Sfrp5 deficiency in combination with a high-fat, high-sucrose diet impaired insulin sensitivity, whereas administration of Sfrp5 reduced adipose tissue inflammation, hyperglycaemia and hepatic steatosis (10). Wnt5a has been mostly studied in the context of diverse developmental processes, but has also been implicated in the development of atherosclerosis and other inflammation-related conditions (11,12). In a recent study Wnt5a was shown to be expressed in adipose tissue macrophages in obese and type 2 diabetes patients and to be involved in the inhibition of the adipogenesis (13). Furthermore, Wnt5a is also expressed in endothelial cells and atherosclerotic lesions (14,15) and induces the expression and release of proinflammatory cytokines (15), an effect which is blocked by Sfrp5 (10). Therefore, it can be postulated that the Sfrp5/Wnt5a ratio may contribute to the development of inflammation-related comorbities of obesity such as insulin resistance, type 2 diabetes and atherosclerosis.

Interestingly, Sfrp5 shares several characteristics with adiponectin in mice. Both adipokines are expressed in white adipose tissue, and their production is lower in obese mice. Furthermore, adiponectin and Sfrp5 have anti-inflammatory properties and improve insulin sensitivity (10,16,17). In humans, previous studies have shown that high levels of adiponectin are associated with a reduced risk of type 2 diabetes (18,19) and that serum adiponectin levels are upregulated by coffee, a beverage containing many anti-inflammatory and anti-oxidative compounds (20).

So far, data on circulating concentrations of Sfrp5 and Wnt5a in humans are not available. Therefore, the aims of the study were (i) to characterise the distribution of absolute concentrations of Sfrp5 and Wnt5a in human serum, (ii) to investigate the association between Sfrp5, Wnt5a, their ratio and adiponectin in relation to

demographic, anthropometric, metabolic and immunological variables, and (iii) to test whether Sfrp5, Wnt5a and their ratio are regulated by coffee drinking.

Subjects and Methods

Study population and design

This study is based on a single-blind (investigator), 3-stage coffee intervention study (clinical trial registration number: ISRCTN12547806) in which 47 individuals refrained from coffee drinking for one month. In the second month, they consumed 4 cups and in the third month 8 cups of coffee per day (1 cup = 150 ml). Serum samples were collected at the end of each month before and after a 2-hour (75 g) oral glucose tolerance test (OGTT). Signed informed consent was obtained from all participants, and this study has been conducted according to the principles expressed in the Declaration of Helsinki. The study population and study design were previously described in detail (20).

Measurement of metabolic parameters and of immune mediators

The measurement of plasma glucose, serum insulin, lipids and immune mediators has been described before in detail (20). We calculated the following parameters obtained from OGTT blood samples for the assessment of correlations between adipokines and glucose metabolism: (i) fasting glucose [mmol/l]; (ii) fasting insulin [µU/ml]; (iii) homoeostasis model assessment-insulin resistance (HOMA-IR) calculated as (fasting glucose [mmol/l] * fasting insulin [µU/ml]) / 22.5 (21); (iv) homoeostasis model assessment-beta-cell function (HOMA-B) calculated as (fasting insulin [µU/ml] * 20) / (fasting glucose [mmol/l] – 3.5) (20); (v) quantitative insulin sensitivity check index (QUICKI) calculated as (1/(log fasting glucose [mmol/l] + log fasting insulin [µU/mol]) (22); (vi) Stumvoll index calculated as 0.226

- (0.0032 * BMI [kg/m^2]) - (0.0000645 * 120 min insulin [pmol/l] -(0.00375 * 90 min glucose [mmol/l]) (23); (vii) insulinogenic index calculated as (30 min insulin – fasting insulin) [µU/ml] / (30 min glucose – fasting glucose) [mmol] (24); (viii) area-under-the-curve for glucose using integration of glucose levels at 0, 30, 60, 90 and 120 min during the OGTT.

Measurement of human Sfrp5 and Wnt5a serum concentrations

Serum concentrations of Sfrp5 and Wnt5a were measured with enzyme-linked immunosorbent assays (ELISAs) (USCN Life Science, Wuhan, PR China). Samples from the same study participants were always analysed on the same plate. Most sera were diluted 1:10 for the Sfrp5 measurements, whereas mainly undiluted sera were used for the Wnt5a assay. Intra-assay and inter-assay CVs were 13.1% and 18.7% for Sfrp5 and 3.1% and 11.9% for Wnt5a, respectively. The detection limits were 0.78 ng/ml for Sfrp5 and 0.02 ng/ml for Wnt5a.

Statistical analyses

Associations with anthropometric and demographic variables, markers of glucose and lipid metabolism as well as markers of subclinical inflammation and oxidative stress were assessed with Pearson correlation coefficients for log-transformed Sfrp5 and adiponectin values (also using log transformed data for all aforementioned variables without normal distribution) and by Spearman correlation coefficients for Wnt5a and the ratio Sfrp5/Wnt5a that could both not be approximated to a normal distribution by log transformation.

The impact of coffee on Sfrp5 and Wnt5a levels and on the Sfrp5/Wnt5a ratio was tested using repeated measures ANOVA for log-transformed Sfrp5 values and Wilcoxon signed rank test for untransformed Wnt5a values and for the ratio

Sfrp5/Wnt5a. Differences in Sfrp5 and Wnt5a levels between subjects with low HOMA-IR (≤3.93) and high HOMA-IR (>3.93) and differences in their ratio were analysed using a paired t-test for log-transformed Sfrp5 levels and the Wilcoxon signed-rank test for untransformed Wnt5a values and for the ratio Sfrp5/Wnt5a. The threshold value for HOMA-IR (median value) was chosen based on our recently published results from this study to allow comparison of the outcomes.

The level of significance was 0.05. Statistical analyses were performed by using GraphPad Prism (version 4; GraphPad Software Inc, La Jolla, CA).

Results

Study population

People at high risk of type 2 diabetes, but currently free of type 2 diabetes, were recruited to the study. Baseline characteristics of the study population were described previously (20) and are given as Supplementary Table S1. The mean age was 54 years, 77% of the study population were female. Most were overweight (52%) or obese (41%), and the mean HOMA-IR of 4.2 indicated a relative high degree of insulin resistance.

Serum concentrations of Sfrp5, Wnt5a and their ratio in humans

Sfrp5 concentrations ranged from 96.1 ng/ml to 4 056 ng/ml. Most study participants had Sfrp5 serum concentrations between 200-800 ng/ml (Fig. 1A). 80.9 % of the study participants had Wnt5a serum concentrations below the detection limit. These individuals were assigned a Wnt5a level of 0.01 ng/ml (i.e. half of the detection limit) for graphic representation and further analyses. Only two samples showed Wnt5a levels above 1 ng/ml (Fig. 1B). The ratio Sfrp5/Wnt5a ranged from 120 to 239 478, whereby most of these ratios were below 100 000

(Fig. 1C). No differences in concentrations of Sfrp5, Wnt5a and the ratio Sfrp5/Wnt5a between women and men were found (all $p>0.05$). Moreover, there were also no differences regarding these parameters between lean, overweight and obese individuals (data not shown).

Associations of serum Sfrp5, Wnt5a and adiponectin with measures of insulin sensitivity and insulin secretion

None of the measured adipokines showed significant correlations with age, measures of obesity and blood pressure (all $p>0.05$). Sfrp5 and Wnt5a showed no significant correlations with adiponectin (both $p>0.05$). Sfrp5 was directly associated with HOMA-IR ($r=0.32$; $p<0.05$), whereas adiponectin was inversely associated with HOMA-IR ($r=-0.50$; $p<0.001$). In addition, high concentrations of adiponectin, but not of Sfrp5, were inversely correlated with further measures of insulin resistance and beta-cell function: fasting insulin ($r=-0.51$, $p<0.001$), HOMA-B ($r=-0.44$, $p<0.01$), QUICKI ($r=0.47$, $p<0.01$), Stumvoll index ($r=0.36$, $p<0.05$) and insulinogenic index ($r=-0.34$, $p<0.05$). Similar to adiponectin, Wnt5a showed an inverse association with HOMA-B ($r=-0.33$, $p<0.05$) (Table 1).

Associations of adipokines with lipids

Adiponectin was directly correlated with apolipoprotein A-I, the main component of HDL cholesterol ($r=0.56$, $p<0.001$), and inversely correlated with the ratio apolipoprotein B/apolipoprotein A-I ($r=-0.46$, $p<0.01$) and oxidized LDL ($r=-0.30$, $p<0.05$), whereas no significant correlations with lipids were seen for Sfrp5, Wnt5a or their ratio (Table 1).

Associations of adipokines with oxidative stress and markers of subclincial inflammation

Sfrp5 showed direct correlations with markers of oxidative stress, 8-isoprostane ($r=0.44$, $p<0.01$) and nitrotyrosine ($r=0.52$, $p<0.001$). Wnt5a was directly correlated with MIF ($r=0.30$, $p<0.05$). The ratio Sfrp5/Wnt5a was directly associated with IL-18 ($r=0.39$, $p<0.01$). In contrast to these findings, there were no significant correlations between adiponectin and all measured markers of subclinical inflammation and oxidative stress (all $p>0.05$) (Table 1).

Impact of coffee consumption on adipokines

The daily consumption of 4 or 8 cups of coffee did not alter Sfrp5 concentrations and the Sfrp5/Wnt5a ratio (Fig. 2A, Fig. 2C). In contrast, serum concentrations of adiponectin increased by 8% after consumption of 8 cups compared to 0 cups of coffee per day as previously reported (20). Serum Wnt5a concentrations were reduced by 42.6% after drinking 4 cups of coffee compared to 0 cups of coffee per day ($p = 0.01$), but serum Wnt5a concentrations did not change significantly after the consumption of 8 cups compared to 0 cups of coffee per day (Fig. 2B).

We have earlier shown that the effect of coffee consumption on adiponectin was more pronounced in individuals with high versus low insulin resistance (20). Therefore, we performed analogous exploratory analysis in this study. After consumption of 0, 4 and 8 cups of coffee per day Sfrp5 levels tended to be higher in participants with high HOMA-IR compared to subjects with low HOMA-IR. This finding was only significant after the consumption of 4 cups compared to 0 cups of coffee per day ($p<0.05$) (Fig. 3A). In contrast, Wnt5a tended to be lower in individuals with high HOMA-IR compared to subjects with low HOMA-IR, but differences were not statistically significant. (Fig. 3B). However, the levels of Sfrp5

and Wnt5a did not change as a response to coffee consumption in either subgroup of insulin resistance. Thus, we found no evidence of an interaction with insulin resistance regarding the effect of coffee consumption on serum levels of both adipokines.

Discussion

Serum concentrations of Sfrp5 and Wnt5a in a human study population

Here we report that in a mostly overweight and obese study population, serum Sfrp5 concentrations range from about 100 to 4 000 ng/ml. This is about 10 times lower than circulating adiponectin concentrations. Wnt5a levels are considerably lower and not detectable in about 80% of this population given a detection limit of 0.02 ng/ml. Because of the great variability of Sfrp5 and Wnt5a in human serum, the Sfrp5/Wnt5a ratio exhibited a substantial range from about 100 to 250 000. To the best of our knowledge, this is the first study to characterise circulating concentrations of Sfrp5 and Wnt5a in a human population. Ouchi et al. focused in their study on expression levels of these novel adipokines in adipose tissue of different mouse models (10), so that there are no comparable data from rodents.

Associations of Sfrp5, Wnt5a and adiponectin with demographic, anthropometric, metabolic and immunological variables

Ouchi et al. also reported anti-inflammatory and insulin-sensitising properties of Sfrp5 in mice (10), so that we hypothesised that associations between Sfrp5 and demographic, anthropometric, metabolic and immunological variables would be similar as for adiponectin. However, our study demonstrates that Sfrp5 and

adiponectin levels differ in many respects regarding these associations in our human study sample.

Most importantly, we found the expected inverse associations with insulin resistance for adiponectin, but not for Sfrp5. In contrast, Sfrp5 even exhibited a direct correlation with HOMA-IR. Since data from the OGTT with repeated measurements of glucose (0, 30, 60, 90, 120 min) and insulin (0, 30, 120 min) were available, we calculated the area under the glucose curve, indices of insulin resistance and beta-cell function based on fasting glucose and insulin values (HOMA-IR, QUICKI, HOMA-B) and indices that take into account the dynamic conditions of an OGTT (Stumvoll index, insulinogenic index). We cannot exclude that the selection of our study population (mainly obese individuals who were more insulin resistant than a sample from the general population) could have contributed to this finding for Sfrp5, but even if this were the case it seems that in humans any inverse association between Sfrp5 and insulin resistance is most likely less pronounced than that of adiponectin.

We found no associations between both Sfrp5 and adiponectin with markers of subclinical inflammation. These data indicate that the anti-inflammatory properties of both adipokines may be more relevant locally, but are less evident when measured at a systemic level. Sfrp5 also differed from adiponectin in so far as it associated positively with markers of oxidative stress including oxidised LDL. Given our study design we do not know whether this finding is due to the fact that expression of Sfrp5 is part of an oxidative stress response or whether Sfrp5 is induced to counterregulate oxidative stress. As expected, adiponectin was directly correlated with apolipoprotein (apo) A-I, the main component of HDL cholesterol, and inversely with the ratio of apoB/apoA-I, whereas these associations were absent for Sfrp5.

Based on previous work on the potential relevance of Wnt5a for the development of atherosclerosis it can be postulated that Wnt5a levels should be correlated with biomarkers of cardiovascular risk (14,15). However, apart from correlations with HOMA-B and MIF serum concentrations of Wnt5a were largely uncorrelated with all variables measured. The fact that about 80% of all tested sera gave no measurable Wnt5a data may have contributed to this null finding, and it can be assumed that more sensitive immunoassays may be required to investigate determinants of circulating Wnt5a levels more thoroughly.

The high number of undetectable Wnt5a concentrations also means that the findings regarding the ratio of Sfrp5/Wnt5a have to be interpreted with caution. The only statistically significant correlation of this ratio in our study was with IL-18, a proinflammatory cytokine that is expressed at low levels in human adipocytes (7) and is associated with insulin resistance and risk for type 2 diabetes (25-27). Due to the high number of tests performed we cannot exclude the possibility of a chance finding. In any case, this association refutes rather than confirms our initial hypothesis that Sfrp5 and the ratio Sfrp5/Wnt5a may be correlates of insulin sensitivity in humans.

Impact of coffee consumption on Sfrp5 and Wnt5a

Lifestyle factors such as dietary habits are described to have an important impact on adipokine concentrations. We have previously shown in the same population that high consumption of coffee decreased serum concentrations of the proinflammatory cytokine IL-18 and the oxidative stress marker 8-isoprostane, whereas adiponectin serum concentrations were increased (20). However, coffee consumption did neither affect Sfrp5 levels nor the Sfrp5/Wnt5a ratio. Serum concentrations of the proinflammatory Wnt5a were significantly decreased at one

timepoint during the study only. As this finding is based on a study population with many Wnt5a levels below the detection limit and as there was no clear dose-response relationship, it is difficult to interpret and at best hypothesis-generating. A decrease of the pro-inflammatory adipokine Wnt5a after coffee consumption would fit to the mainly antiinflammatory effects of coffee, but further studies are required to evaluate the potential impact of coffee on proteins of the Wnt system.

Again based on our previous study (20) in which we reported that individuals with high degree of insulin resistance responded to coffee consumption by an upregulation of adiponectin, whereas no effects were seen in insulin-sensitive individuals, we performed analagous analyses for Sfrp5 and Wnt5a, but found no effect of coffee in either group on these adipokines. This underlines that the regulation of adiponectin and Sfrp5 may differ in humans.

Strengths and limitations of the study

The study participants were well characterised including detailed information on anthropometric, demographic, metabolic and inflammatory parameters. Furthermore, the OGTT allowed for the analysis of fasting and post-challenge glucose.

Our study had several limitations. The study was designed as a one-group study without randomisation, blinding of the participants and placebo control. Most study participants were female and obese so that our results may not be fully generalisable to men or lean populations. In addition, the observation period may be too short to reveal clinically relevant changes in metabolic or immunological parameters that may be conferred by long-term coffee consumption. The sensitivity of the Wnt5a assay (to our knowledge currently the only commercially available immunoassay for this protein) precluded a more precise investigation of

associations between Wnt5a serum levels and other variables of interest. Finally, we performed multiple tests to assess determinants of Sfrp5, Wnt5a and their ratio so that our data should be replicated in larger studies.

Conclusion

Taken together, Sfrp5 was directly associated with markers of oxidative stress, but not with insulin sensitivity and an anti-atherogenic lipid profile like adiponectin and Wnt5a. Sfrp5 was also not upregulated by coffee drinking like adiponectin, whereas Wnt5a was downregulated. Associations between Sfrp5 and obesity-associated subclinical inflammation, insulin resistance and adverse lipid profile seem to differ between humans and mice, and further studies are required to evaluate whether the balance between Sfrp5 and Wnt5a contributes to the development of insulin resistance and type 2 diabetes in humans.

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Tables

Table 1 Correlations between fasting serum concentrations of Sfrp5, Wnt5a and adiponectin as well as the ratio Sfrp5/Wnt5a and anthropometric, demographic, metabolic and immunological variables at baseline.

Variables		Sfrp5	Wnt5a	Adiponectin	Sfrp5/Wnt5a
Anthropometric and demographic	**Age**	- 0.15	- 0.14	0.25	0.07
	Weight	0.15	- 0.19	- 0.16	0.22
	BMI	0.08	- 0.19	- 0.14	0.20
	Waist cirumference	0.12	- 0.08	- 0.26	0.07
	Systolic blood pressure	- 0.08	- 0.04	0.21	- 0.09
	Diastolic blood pressure	- 0.09	- 0.04	0.13	- 0.16
Glucose metabolism	**Glucose**	0.26	0.03	- 0.07	0.04
	Insulin	0.27	- 0.27	**- 0.51*** **	0.25
	HOMA-IR	**0.32***	- 0.27	**- 0.50*** **	0.25
	HOMA-B	0.14	**- 0.33***	**- 0.44****	0.28
	QUICKI	- 0.23	0.27	**0.47****	- 0.25
	Stumvoll index	- 0.15	0.14	**0.36***	- 0.11
	Insulinogenic index	- 0.07	- 0.08	**- 0.34***	- 0.03
	Glucose (AUC)	0.20	- 0.13	- 0.25	0.13
Lipids	**Apo A-I**	- 0.14	0.01	**0.56*** **	-0.05
	Apo B	0.19	0.17	- 0.24	0.05
	Apo B/Apo A-I	0.27	0.12	**- 0.46****	0.04
	Oxidized LDL	0.27	0.07	**- 0.30***	0.16
Subclinical inflammation	**CRP**	0.10	- 0.12	- 0.18	0.10
	SAA	- 0.01	- 0.05	- 0.09	0.02
	IL-6	0.16	0.01	- 0.04	0.03
	IL-18	0.24	- 0.21	- 0.13	**0.39****
	MIF	0.07	**0.30***	0.21	- 0.12
	IL-1Ra	0.02	0.04	- 0.27	- 0.04
	Sfrp5	**1*** **	- 0.14	- 0.09	**0.76*** **
	Wnt5a	- 0.14	**1*** **	0.08	**- 0.68*** **
	Adiponectin	- 0.09	0.08	**1*** **	- 0.06
	Leptin	- 0.08	0.04	0.03	- 0.11
Oxidative stress	**8-Isoprostane**	**0.44****	0.21	0.08	0.14
	Nitrotyrosine	**0.52*** **	- 0.03	0.14	0.28

Correlations are given as Pearson correlation coefficients (r) for log-transformed values of Sfrp5 and adiponectin and as Spearman correlation coefficients (r) for Wnt5a and the ratio Sfrp5/Wnt5a. *p<0.05; **p<0.01; ***p<0.001. Significant correlations are highlighted using bold print. Apo, apolipoprotein; AUC, area under the curve; BMI, body mass index; CRP, C-reactive protein; HOMA-B, homeoestasis model assessment of beta cell function; HOMA-IR, homeoestasis model assessment of insulin resistance; IL, interleukin; IL-1Ra, Interleukin-1 receptor antagonist; LDL, low-density lipoprotein cholesterol; MIF, macrophage migration inhibitory factor; QUICKI, quantitative insulin sensitivity check index; SAA, serum amyloid A; Sfrp5, secreted frizzled-related protein 5.

Supporting Information Legend

Table S1 (Supplement) Baseline characteristics of the study population

Variable	Study population (N = 47)
Anthropometric and demographic	
Sex (N (%)]	
Men	11 (23)
Women	36 (77)
Age (years)	54.0 ± 9.0
BMI (kg/m^2)	29.2 ± 4.6
Waist circumference (cm)	98.1 ± 10.5
Systolic blood pressure (mm Hg)	140.5 ± 15.3
Diastolic blood pressure (mm Hg)	89.5 ± 9.3
Smoking [N (%)]	
Yes	2 (4)
No	45 (96)
Glucose metabolism	
Glucose (mmol/l)	5.8 ± 0.5
Insulin (µU/ml)	15.9 ± 6.7
HOMA-IR	4.2 ± 1.8
HOMA-B	139.9 ± 63.0
QUICKI	0.5 ± 0.1
Stumvoll index	0.08 ± 0.03
Insulinogenic index	15.6 (10.4, 27.9)
Glucose area-under-the-curve (AUC)	956.9 ± 184.8
Lipid metabolism	
Apo A-I (g/l)	1.6 ± 0.3
Apo B (g/l)	1.1 ± 0.2
Apo B / Apo A-I	0.7 ± 0.2
Oxidised LDL (U/ml)	100.7 ± 26.7
Subclinical inflammation	
CRP (mg/l)	1.2 (0.5, 1.9)
SAA (mg/l)	3.2 (1.9, 6.6)
IL-6 (pg/ml)	1.1 (0.3, 1.8)
IL-18 (pg/ml)	128.0 ± 55.7
MIF (ng/ml)	10.5 (7.8, 14.7)
IL-1Ra (pg/ml)	296 (208, 472)
Sfrp5 (ng/ml)	500 (336, 856)
Wnt5a (ng/ml)	0.2 ± 0.7
Adiponectin (ng/ml)	7 957 (6 317, 10 901)
Leptin (ng/ml)	32.9 + 21.5
Oxidative stress	
8-Isoprostane (pg/ml)	79.6 (39.4, 151.6)
Nitrotyrosine (nmol/l)	42.4 (5.3, 145.4)

Data are given as N and %, mean ± SD or median (25th, 75th percentile).

Figures

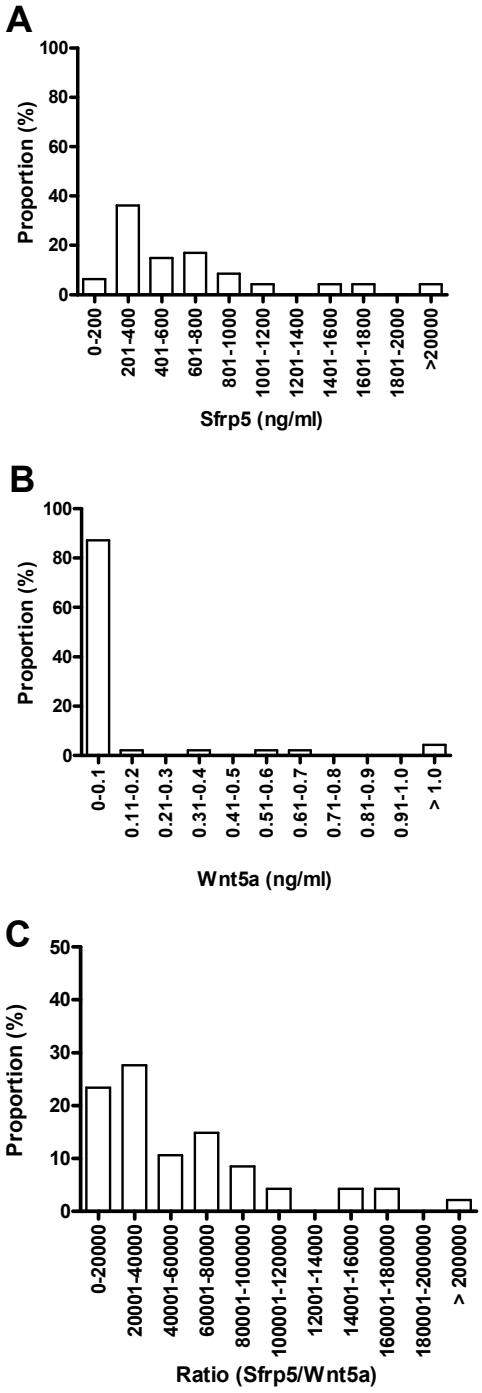

Figure 1 Distribution of Sfrp5 (A) and Wnt5a (B) serum concentrations and the ratio Sfrp5/Wnt5a (C) in the study population. Serum concentrations are given as ng/ml, the number of study participants in percent.

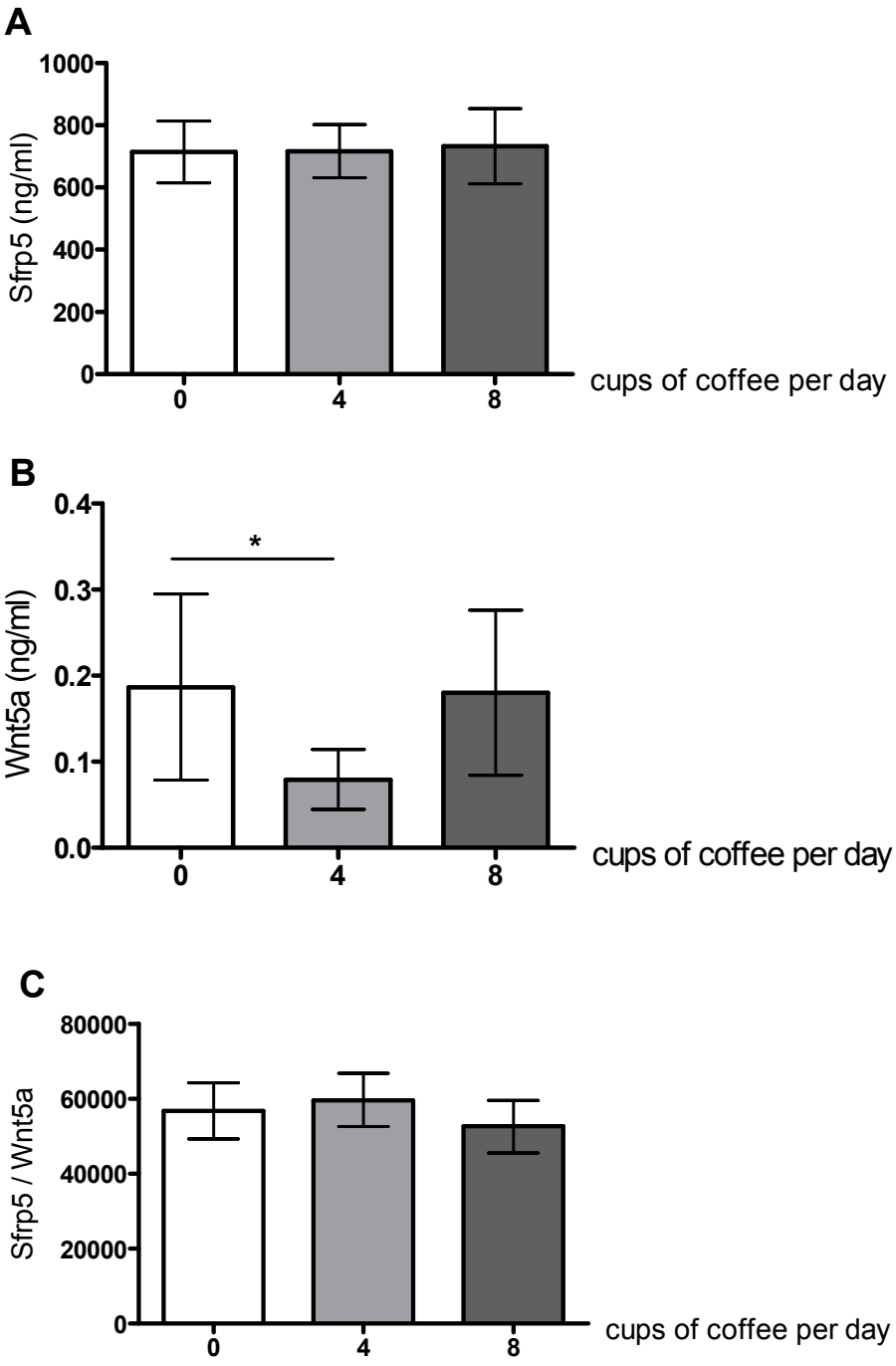

Figure 2 Fasting serum concentrations of Sfrp5 (A), Wnt5a (B) and the ratio Sfrp5/Wnt5a (C) after consumption of 0, 4 and 8 cups of coffee per day.

Data are given as mean ± SEM. *p<0.05.

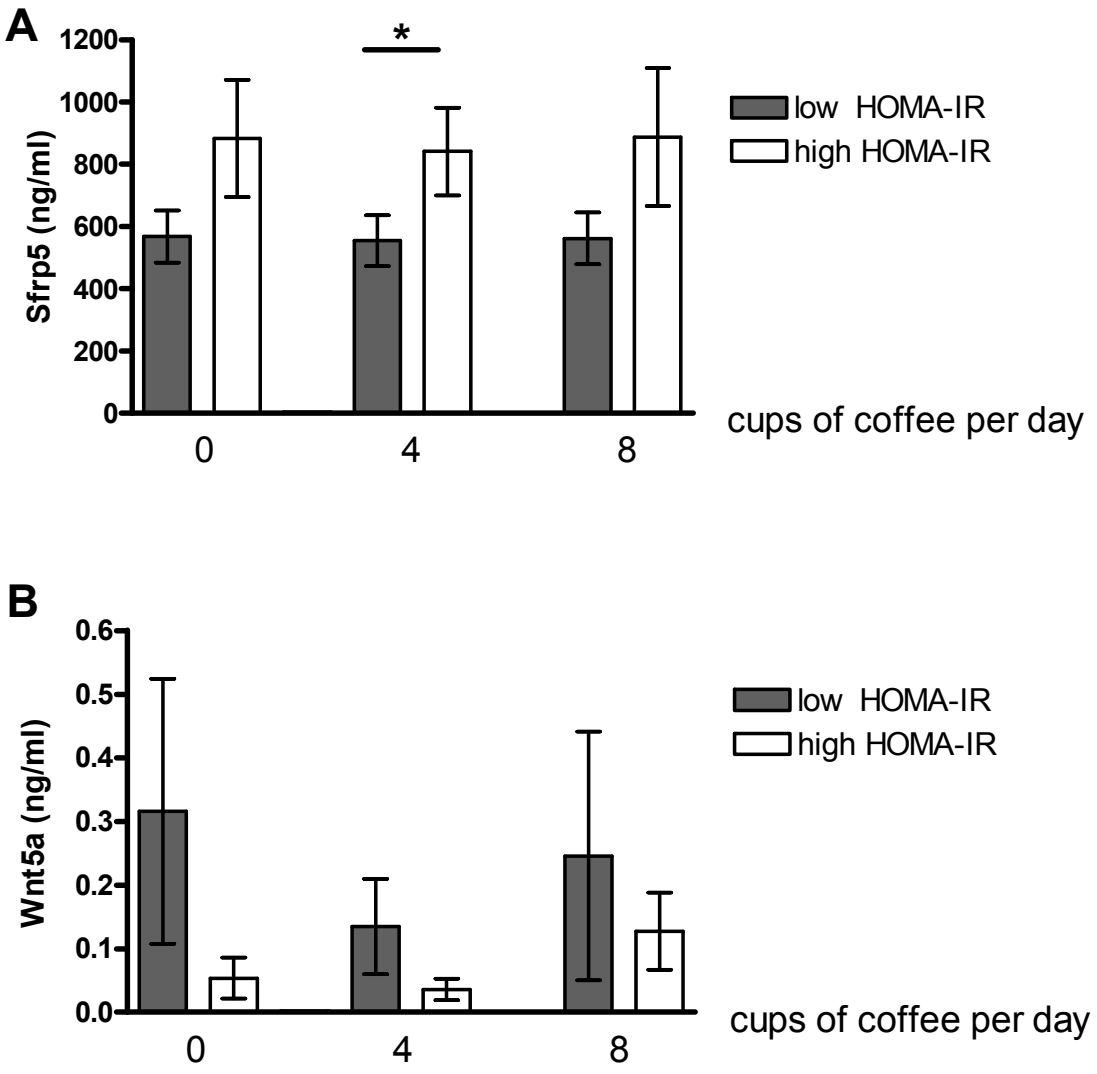

Figure 3 Influence of insulin resistance on fasting serum concentrations of Sfrp5 (A) and Wnt5a (B) and their regulation by coffee. The study population was divided in two subgroups according to the median of HOMA-IR. The first subgroup had low insulin resistance (HOMA-IR $\leq$3.93) and the second subgroup had high insulin resistance (HOMA-IR >3.93). Data are given as mean $\pm$ SEM. *p<0.05.

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Contribution: Total: 90%

Conceived / designed experiments:	80%
Performed experiments:	100%
Analysed data:	90%
Contributed to discussion:	80%
Wrote the manuscript:	70%
Reviewed / edited manuscript:	60%

Author: 1st author

Chapter 6

General Discussion

The role of pro-inflammatory immune mediators in the development of type 2 diabetes is well characterised, but there are still wide gaps in our knowledge of the role of anti-inflammatory immune mediators in this context. This doctoral thesis focused on the role of four anti-inflammatory immune mediators in the development of type 2 diabetes: interleukin-1 receptor antagonist (IL-1Ra), adiponectin, macrophage inhibitory cytokine-1 (MIC-1) and secreted frizzled-related protein 5 (Sfrp5). The main findings of these investigations are:

- IL-1Ra levels were elevated (302 vs. 244 pg/ml, $p<0.0001$) and adiponectin levels were reduced (7141 vs. 8818 ng/ml, $p<0.0001$) in individuals who subsequently developed type 2 diabetes compared to those who remained diabetes-free 13 years before type 2 diabetes diagnosis.

- In subjects who subsequently developed type 2 diabetes, IL-1Ra levels steeply increased 6 years before type 2 diabetes diagnosis (13-16 pg/ml per year compared to <1.5 pg/ml per year for non-cases, $p<0.0001$).

- Adiponectin levels decreased more strongly in female cases compared to non-cases (slope difference -1.1% per year, $p=0.004$) than in male cases (no significant slope difference compared to non-cases). We also found that early-onset cases of type 2 diabetes (<52 years of age at the time of diagnosis) had a steeper decline of adiponectin levels than non-cases (slope difference -1.8% per year, $p=0.016$), whereas no significant slope difference was observed for late-onset cases (≥52 years of age at the time of diagnosis) of type 2 diabetes.

- In contrast to adiponectin, MIC-1 concentrations were increased in individuals before type 2 diabetes diagnosis in comparison with subjects who remained diabetes-free (537 pg/ml vs 500 pg/ml, $p = 0.0044$), but MIC-1 was not independently associated with incident type 2 diabetes.

- Sfrp5 was directly correlated with HOMA-IR ($r=0.32$, $p<0.05$) and oxidative stress (8-isoprostane: $r=0.44$, $p<0.01$; nitrotyrosine: $r=0.52$, $p<0.001$), and therefore associations with metabolic and immunological variables differed from those found for adiponectin in humans.

6.1 Trajectories of IL-1Ra and adiponectin before the manifestation of type 2 diabetes

Both anti-inflammatory immune mediators, IL-1Ra and adiponectin, were investigated in the serum of study participants of a prospective case-cohort study within the Whitehall II cohort who were diabetes-free at the beginning of the study. All analyses were based on 335 individuals who subsequently developed type 2 diabetes (cases) and 2,475 subjects who remained diabetes-free (non-cases). We used repeated measurements at up to three different timepoints for each subject to determine the course of the levels of IL-1Ra and adiponectin in individuals before the manifestation of type 2 diabetes in comparison with subjects who remained diabetes-free.

Determinants of systemic levels of IL-1Ra and adiponectin

As the analyses of the association between outcome (here: incident type 2 diabetes) and exposition (here: IL-1Ra or adiponectin) could be influenced by confounders, it is important to identify the determinants of both, IL-1Ra and adiponectin levels, within the Whitehall II study. Adjustments for these determinants might reduce the risk of over- or underestimation of differences between trajectories of cases and non-cases for IL-1Ra and adiponectin.

Both immune mediators were higher in women than in men suggesting that sex hormones and sex-specific differences in the distribution of adipose tissue might influence the regulation of IL-1Ra and adiponectin. In adult men, the administration of testosterone decreases the levels of IL-1β (175) and adiponectin (176). Currently, there are no data available on the impact of sex hormones on IL-1Ra. However, IL-1Ra antagonises IL-1β and the levels of both immune mediators are likely to be tightly linked to each other. Therefore, changes in the levels of IL-1β might also influence the levels of IL-1Ra. Moreover, the distribution of adipose tissue is different between women and men. Women have more subcutaneous adipose tissue, whereas men have more visceral adipose tissue (177). As both, IL-1Ra and adiponectin are released from adipose tissue, the different distribution of adipose tissue might also have an impact on the sex-specific differences in the levels of both immune mediators. Serum adiponectin showed a negative correlation with subcutaneous and visceral fat mass. However, the correlation was

stronger with visceral fat mass in patients with type 2 diabetes (178). In obese subjects, visceral adipose tissue contained a higher amount of IL-1Ra compared to subcutaneous adipose tissue (179,180).

The impact of ethnicity on IL-1Ra and adiponectin could not be evaluated because of the low number of non-white individuals in this cohort. However, there are some other determinants that influence the levels of IL-1Ra and adiponectin. The present data of this thesis showed that IL-1Ra was positively associated with age, indices of obesity (BMI, waist circumference), blood pressure, levels of fasting insulin and 2-h glucose. Unpublished data from the Whitehall II study showed that adiponectin was positively associated with age (r=0.06) and showed inverse correlations with BMI (r=-0.22), waist circumference (r=-0.40), blood pressure (systolic: r=-0.09; diastolic: r=-0.15), fasting blood glucose levels (r=-0.19) and fasting serum insulin levels (r=-0.29) (all $p<0.0001$).

6.1.1 IL-1Ra trajectories before type 2 diabetes manifestation

Our findings demonstrated that serum levels of IL-1Ra were increased in individuals 13 years before type 2 diabetes diagnosis (cases) compared to those who remained diabetes-free (non-cases). IL-1Ra levels steeply increased in individuals about 6 years before type 2 diabetes diagnosis, whereas IL-1Ra levels of subjects who remained diabetes-free were unaltered.

Our hypothesis was that IL-1Ra levels increase about 5 years before type 2 diabetes diagnosis because of previously described changes of insulin sensitivity and β-cell function at this time-point. Our findings are in line with our hypothesis and previous data from our research group on IL-1Ra in a prospective, nested case-control study within the Whitehall II cohort that showed an association of elevated levels of IL-1Ra with a higher risk of type 2 diabetes (116).

Adjustments of the IL-1Ra trajectories

The trajectories of IL-1Ra were adjusted for age, sex and ethnicity. The additional adjustment for measures of obesity (BMI and waist circumference) attenuated, but did not abolish the differences in IL-1Ra between cases and non-cases. Adipocytes are one important cell type for the release of IL-1Ra, but there also other sources of IL-1Ra like β-cells (131), epithelial cells (122,123) and

hepatocytes (129). Therefore, obesity may explain only a small proportion of the variance of IL-1Ra levels.

The additional adjustment for fasting insulin had hardly an impact on the differences between the IL-1Ra levels of subjects who subsequently developed type 2 diabetes compared to those who remained diabetes-free 6 to 13 years before type 2 diabetes diagnosis. However, the steep increase in IL-1Ra in individuals six years before the diagnosis was substantially attenuated after adjustment for fasting insulin levels. This is in line with unpublished data on the trajectories of fasting insulin among the same study cohort indicating that fasting insulin steeply increased in individuals about 7 years before type 2 diabetes diagnosis, whereas fasting insulin levels were unaltered in subjects who remained diabetes-free. In addition, the present data showed that IL-1Ra showed a positive correlation with fasting insulin ($r=0.283$, $p<0.0001$). These findings might indicate that IL-1Ra and fasting insulin levels are tightly linked to each other. Breakpoints in the trajectories for IL-1Ra and fasting insulin levels were found at almost the same time-point, so that we cannot determine the direction of causality between IL-1Ra and fasting insulin. So far, there are no data on the interaction between IL-1Ra and fasting insulin.

Comparison between IL-1Ra trajectories with trajectories of insulin sensitivity, β-cell function and glycaemia

The trajectories of insulin sensitivity, β-cell function, fasting and 2-h glucose were also investigated for the same study participants (37). Insulin sensitivity steeply decreased and the β-cells obviously tried to compensate this reduced insulin action by increased insulin secretion at almost the same time as IL-1Ra steeply increased in subjects to develop type 2 diabetes; an attempt that failed about 2 years before diagnosis because of decreasing β-cell function. Moreover, 2-h glucose levels steeply increased at about the same time-point as IL-1Ra (37) suggesting that IL-1Ra and glucose metabolism are tightly linked. However, our data cannot disentangle whether increased levels of IL-1Ra are a cause or a consequence of these metabolic changes that are all known to contribute to the development of type 2 diabetes.

The role of IL-1β in insulin resistance and β-cell dysfunction

IL-1Ra is characterised as an anti-inflammatory immune mediator that is protective against type 2 diabetes and antagonises the pro-inflammatory IL-1β (117,118,138). As the balance between IL-1Ra and IL-1β may be important for the severity of several inflammatory diseases such as type 2 diabetes (181), it would be interesting to calculate the ratio between IL-1Ra and IL-1β. So far, there is no sufficiently sensitive method to determine the IL-1β protein levels in human serum because IL-1β concentrations are predominantly below the detection limits of available assays. However, there are some data on the deleterious effects of the pro-inflammatory IL-1β on the two most important pathogenic mechanisms promoting the development of type 2 diabetes: insulin resistance and β-cell dysfunction.

Insulin sensitivity is decreased by IL-1β by four possible mechanisms in murine and human adipocytes and rat hepatocytes *in vitro*: (1) interference with insulin signalling, (2) suppression of insulin-induced glucose transport, (3) inhibition of lipogenesis and (4) reduction of the release of adiponectin (103-105).

The deleterious effects of IL-1β on β-cells are mediated by three possible mechanisms: (1) impairment of insulin secretion, (2) reduction in β-cell proliferation and (3) promotion of β-cell apoptosis (182).

In addition, IL-1β is described to upregulate the production of other pro-inflammatory immune mediators leading to a subclinical inflammation that in turn might also contribute to the development of type 2 diabetes (136,183).

Triggers of IL-1β production

Several *in vitro* studies showed that IL-1β is released in higher amounts in both β-cells of patients with diabetes (43) and adipose tissue of obese, prediabetic individuals (135). Therefore, the next important question refers to the triggers of the increased production of IL-1β. It could be demonstrated that high levels of glucose (glucotoxicity) and free fatty acids (lipotoxicity) induce the release of (pro)-IL-1β by activation of NF-κB in β-cells (182). Then a structure called “Nlrp3 (nod-like receptor family, pyrin domain containing 3) inflammasome“ as a danger-sensing cytosolic multiprotein complex is responsible for the conversion of the inactive pro-IL-1β into the active IL-1β (184-186).

Blocking the deleterious effects of IL-1β on β-cells and insulin-responsive tissues

As already mentioned, the balance between IL-1β and IL-1Ra has been postulated to be important for the development of type 2 diabetes (131). Increased levels of IL-1β seem to contribute to the development of type 2 diabetes and IL-1β itself induces the production of its antagonist IL-1Ra (135). Another important question refers to the events after blocking IL-1β in knockout mouse models or after the administration of recombinant IL-1Ra. There are several studies on mice lacking one of the important components of the inflammasome that activate IL-1β: the regulatory subunit Nlrp3, the adaptor ASC (apoptosis-associated speck-like protein) or the effector subunit caspase-1 and therefore lacking the activation of IL-1β. It could be shown that these mice after feeding a high-fat diet showed improved glucose homeostasis (184-186), are protected against the development of high-fat diet-induced obesity, have reduced liver triglyceride content, reduced adipocyte size and adipose tissue mass (185,186). Moreover, the pancreatic β-cells of these mice are protected from cell death (187).

In humans, several therapeutic approaches were performed to block IL-1β. In a double-blind, parallel-group trial with 70 patients with type 2 diabetes it could be shown that the administration of the recombinant human IL-1Ra “Anakinra” improved glycaemia and secretory function of the β-cells, in parallel markers of systemic inflammation were decreased. However, the peak levels of IL-1Ra in the serum shortly after daily injections were more than 1000-fold higher in the intervention group than in the placebo group and thus clearly supraphysiological (138). Other possibilities to block the deleterious effects of IL-1β are the application of human monoclonal antibodies to IL-1β or of soluble receptors for IL-1β (183).

The role of IL-1Ra and IL-1β in the development of type 2 diabetes

The findings of previous studies on IL-1β and IL-1Ra could be combined to develop a hypothetical model of the role of IL-1Ra and IL-1β in the development of type 2 diabetes (Fig. 3).

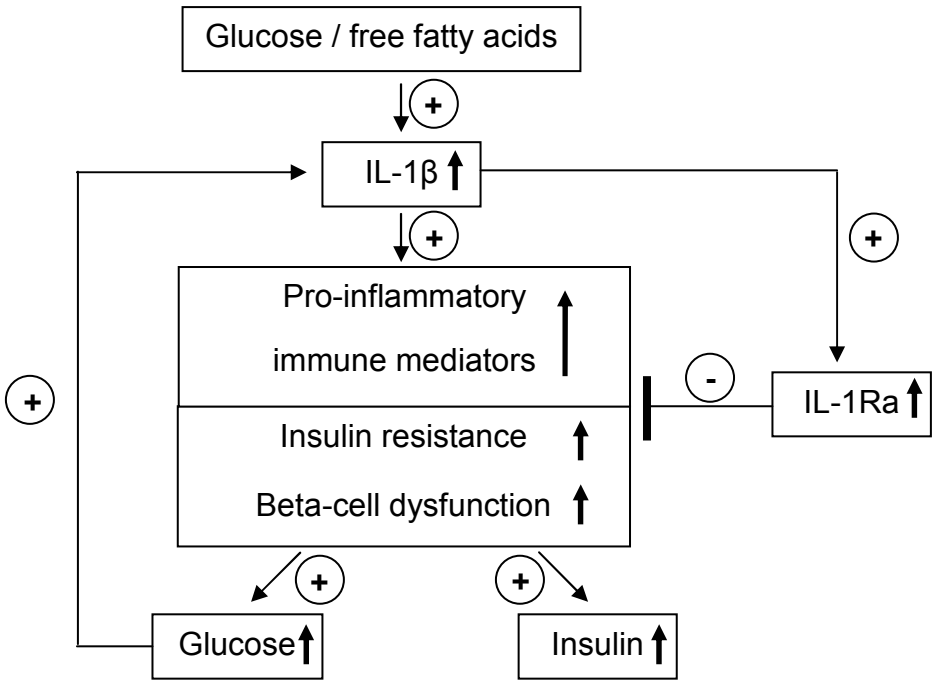

Figure 3: Hypothetical model of the role of IL-1β and IL-1Ra in the development of type 2 diabetes.

Our data are in line with the possible scenario shown in Figure 3. An overload of glucose and free fatty acids due to unfavourable dietary habits leads to elevated levels of IL-1β. This results in (1) subclinical inflammation induced by an upregulation of pro-inflammatory immune mediators and (2) metabolic disturbances caused by insulin resistance and β-cell dysfunction. Both, subclinical inflammation and metabolic disturbances, can contribute to the development of type 2 diabetes. The steep increase in IL-1Ra levels observed in our studies most likely reflects the attempt to counterregulate the deleterious inflammatory and metabolic effects caused by elevated levels of IL-1β. As increasing levels of IL-1Ra could not protect against type 2 diabetes this attempt eventually fails. The metabolic disturbances also lead to increased levels of insulin and glucose. Insulin levels are elevated because of increasing β-cell activity in the attempt to compensate the reduced insulin sensitivity, eventually resulting in a progressive β-cell failure. Glucose levels are increased as a consequence of reduced insulin sensitivity. As glucose has the ability to increase the levels of IL-1β, this results in a vicious circle that might promote the development of type 2 diabetes (Fig. 3).

6.1.2 Adiponectin trajectories before type 2 diabetes manifestation

Our studies revealed that adiponectin levels were decreased in individuals 13 years before type 2 diabetes diagnosis (cases) compared to those who remained diabetes-free (non-cases). Adiponectin trajectories were characterised by either a constant decline in female and male individuals younger than 52 years and female individuals of 52 years and older or by the absence of changes in adiponectin in male individuals of 52 years and older during the 13 years before type 2 diabetes diagnosis. Adiponectin trajectories were unaltered in individuals who remained diabetes-free until the end of the follow-up. There was no marked breakpoint of adiponectin trajectories at any time during the follow-up as observed for IL-1Ra. However, the closer to the time-point of type 2 diabetes diagnosis, the more pronounced differences in adiponectin levels between cases and non-cases were observed.

Our hypothesis was that adiponectin is decreased before type 2 diabetes diagnosis because increased levels of adiponectin might be protective against type 2 diabetes. Our findings are in line with our hypothesis and previous data on the association of adiponectin with the risk of type 2 diabetes in several prospective studies that also indicated that decreased levels of adiponectin are associated with a higher risk of type 2 diabetes (110).

Adjustments of the adiponectin trajectories

In our studies, adiponectin trajectories were adjusted for age and ethnicity. Stratified analyses and additional adjustments showed that adiponectin levels were strongly influenced by sex, obesity and age at diabetes diagnosis.

The sex-stratified trajectories of adiponectin demonstrated that adiponectin concentrations were higher in women than in men 13 years before type 2 diabetes diagnosis. As mentioned before, this could be caused by the different regulation of adiponectin by female and male sex hormones and/or sex-specific distribution of adipose tissue as almost exclusive site of expression and release of this adipokine.

Obesity has a great impact on the trajectories of adiponectin. After adjustment for obesity the differences in adiponectin trajectories between individuals who subsequently developed type 2 diabetes compared to those who remained diabetes-free were substantially attenuated. These differences remained

statistically significant only in women. This in turn points to sex-specific differences in adiponectin regulation by sex hormones and in adipose tissue distribution. The greater impact of obesity on adiponectin trajectories compared to IL-1Ra trajectories might be explained by the site of secretion of these immune mediators. In contrast to IL-1Ra, adiponectin is almost exclusively expressed in adipose tissue. Because of this fact, the impact of obesity on adiponectin might be higher than on IL-1Ra. It is surprising that adiponectin is inversely associated with obesity, because it is only secreted from adipose tissue. However, it could be shown that the expansion of fat led to adipocyte dysfunction characterised by elevated lipolysis, increased levels of pro-inflammatory cytokines and oxidative stress that in turn reduced the levels of adiponectin. Therefore, low adiponectin levels are a hallmark of impaired adipocyte function (188).

Finally, the age at diabetes diagnosis also had an important impact on adiponectin levels. In individuals with an age of diagnosis below 52 years, adiponectin levels decreased more strongly than in subjects of 52 years and older. As obesity has a key role in the regulation of adiponectin levels and explains a substantial part of the slope differences between individuals with early-onset of type 2 diabetes vs. late-onset of type 2 diabetes and individuals who remained diabetes-free, it might be expected that individuals with an early-onset of type 2 diabetes are more obese than the ones with a late-onset of type 2 diabetes. In fact, observations from earlier studies seem to confirm this assumption (189-191).

Comparison of adiponectin trajectories with trajectories of insulin sensitivity, β-cell function and glycaemia

Adiponectin trajectories are characterised by a constant decline without any abrupt change, whereas the trajectories of insulin sensitivity, β-cell function and glycaemia showed marked breakpoints in individuals during the 13 years observation period before type 2 diabetes diagnosis (37). However, adiponectin trajectories were most similar to the trajectories of insulin sensitivity which also decreased during the follow-up.

The role of adiponectin in insulin resistance and β-cell dysfunction

So far, data on adiponectin and the two most important pathogenic mechanisms contributing to the development of type 2 diabetes - insulin resistance and β-cell dysfunction - are somewhat inconsistent.

From previous studies there are some data available on the association between adiponectin and insulin sensitivity and β-cell function in mouse models. In isolated murine islets, adiponectin increased insulin secretion (192,193), but the effect of adiponectin might depend on the glucose levels of insulin-resistant mice. Insulin secretion was inhibited by adiponectin at low glucose levels, whereas insulin secretion was stimulated by adiponectin at high glucose levels. Islets of normal mice were not affected by adiponectin (194). Moreover, adiponectin seemed to protect against pancreatic β-cell apoptosis (193). *In vivo* studies showed that adiponectin knockout mice had moderate insulin resistance (195) and the intravenous injection of adiponectin to wild-type mice increased insulin secretion (192).

In humans, much fewer data on adiponectin are available. *In vitro* experiments with human islets that were exposed to adiponectin, insulin secretion and β-cell apoptosis were not affected (196) but adiponectin was described to have an insulin-sensitising effect on human skeletal muscle from biopsies by blocking the NF-κB inducing kinase (NIK) that induces insulin resistance (197). Several other studies investigated the correlation between adiponectin, insulin resistance and β-cell dysfunction. Adiponectin levels showed an inverse correlation with insulin resistance (198-202), but in some studies this association was limited to overweight individuals (202). A few further studies found an inverse correlation between adiponectin and β-cell dysfunction (198,201) that in some cases was restricted to obese individuals (201).

The relationship between hypoadiponectinaemia and increased risk of type 2 diabetes is complex

Several studies have already shown that decreased levels of adiponectin are associated with an increased risk of type 2 diabetes (110). Interestingly, a recent study demonstrated that the inverse association between adiponectin and the risk of type 2 diabetes levelled off with further increases in adiponectin levels reaching a plateau at concentrations above 10-20 µg/ml (203). This indicates that the role of

adiponectin in type 2 diabetes development is more complex than previously expected. Two recent studies focused on the issue whether the association between hypoadiponectinaemia and incident type 2 diabetes may be explained by insulin resistance and/or hyperinsulinaemia (203,204).

The first study showed that the inverse association between adiponectin and risk of type 2 diabetes was stronger in insulin-sensitive participants indicating that adiponectin is independent of hyperinsulinaemia. In addition, the inverse association between adiponectin and risk of type 2 diabetes was not influenced after adjustment for insulin resistance. Therefore, adiponectin might be independent of hyperinsulinaemia and insulin resistance (203).

The second study investigated the association between genetic variants of the adiponectin gene (*ADIPOQ*) and insulin resistance. The human genetic data do not support a primary role for adiponectin in insulin resistance in humans. It could be shown that the impact of common variants at the *ADIPOQ* locus or mutations in *ADIPOQ* on circulating adiponectin levels was modest, but there was no association with insulin sensitivity (205,206). Because the genetic data do not allow to clarify the direction of causality, it might be possible that changes in adiponectin are rather a consequence of insulin resistance and hyperinsulinaemia (204).

Cook et al. suggested insulin to be able to decrease the levels of adiponectin in humans. So far, the mechanism how insulin may suppress adiponectin is unclear (204). Insulin could suppress the transcription of *ADIPOQ* or induce posttranscriptional and/or posttranslational modifications of the adiponection mRNA/protein (204). Interestingly, in patients with type 1 diabetes who are characterised by low or absent insulin levels, adiponectin levels are increased (207,208). This could be confirmed by directly testing the effect of insulin on adiponectin levels. Healthy individuals received insulin infusions which suppressed adiponectin levels *in vivo* (209,210). These data support the notion that decreased levels of adiponectin might be caused by hyperinsulinaemia. Our studies revealed an inverse correlation between adiponectin and fasting insulin. Moreover, the trajectory analyses among the same study participants showed a constant decline in adiponectin levels during follow-up and a steep increase in fasting insulin seven years before type 2 diabetes manifestation (unpublished data). However, it should be noted that the presence of a breakpoint in the insulin trajectory and its absence

in the adiponectin trajectory are also in line with the interpretation that both adiponectin and fasting insulin are less tightly linked than expected.

The findings on insulin-suppressing effects on adiponectin and the fact that adiponectin seemed to be associated with increased insulin sensitivity and elevated insulin secretion in mouse models and humans might indicate that there might be a bidirectional relationship between adiponectin and insulin action (204). This was already described for sex hormone-binding globulin (SHBG). SHBG is known to be suppressed by insulin in humans and reductions in plasma SHBG are associated with a significantly increased risk of insulin resistance and type 2 diabetes (211-213). Adiponectin and SHBG seem to be putative mediators of insulin resistance both upstream and downstream of insulin action (204).
As there might be a bidirectional relationship between adiponectin and insulin action, the following hypothetical model of the role of adiponectin in the development of type 2 diabetes might be proposed (Fig. 4).

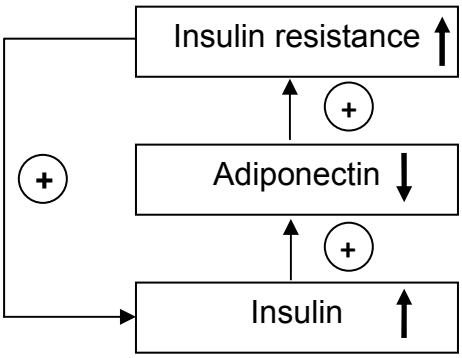

Figure 4: Hypothetical model of the bidirectional relationship between adiponectin and insulin action in the development of type 2 diabetes.

Our data are in line with the following scenario (Fig. 4). Thirteen years before type 2 diabetes diagnosis adiponectin levels were decreased, whereas insulin resistance and fasting insulin were increased. Higher levels of insulin are induced by increased insulin resistance because of the attempt of the β-cells to compensate the decreased insulin sensitivity by elevated insulin secretion. Elevated levels of insulin in turn might promote decreased levels of adiponectin. The bidirectional relationship between adiponectin and insulin seems to be a vicious circle. However, it is still unclear which of these events initiates this process. There might be two possible initiating conditions:

(1) Lifestyle factors such as obesity or certain genetic variants of adiponectin lead to a downregulation of adiponectin levels. Decreased levels of adiponectin are associated with increased insulin resistance. Consequently, insulin levels are increased to compensate the reduced insulin sensitivity and lead to a further downregulation of adiponectin.
(2) Lifestyle factors such as obesity lead to reduced insulin sensitivity resulting in increased insulin levels as compensating attempt. Insulin promotes a downregulation of adiponectin that in turn is associated with insulin resistance.

Based on our findings on the time-course of adiponectin, insulin resistance and fasting insulin the following hypothesis regarding the starting-point of the aforementioned vicious circle of adiponectin and insulin action might be possible: Adiponectin is decreased already 13 years before type 2 diabetes manifestation due to lifestyle factors such as obesity. Although insulin resistance and fasting insulin are also increased 13 years before type 2 diabetes diagnosis, there is a marked breakpoint in their trajectories about five to seven years before the diagnosis of type 2 diabetes. At that time, there is a steep increase in insulin resistance and fasting insulin levels. This might indicate that insulin resistance and the consequent increase of insulin levels are rather influenced by lower levels of adiponectin for years or even more than a decade rather than the opposite way. Therefore, the vicious circle eventually promoting the development of type 2 diabetes might be initiated by adiponectin. However, this hypothesis has to be confirmed by further studies.

The role of IL-1Ra and adiponectin in the development of type 2 diabetes

Based on the findings of previous studies and our trajectory analyses the following model of the role of IL-1Ra and adiponectin in the development of type 2 diabetes could be be proposed (Fig. 5).

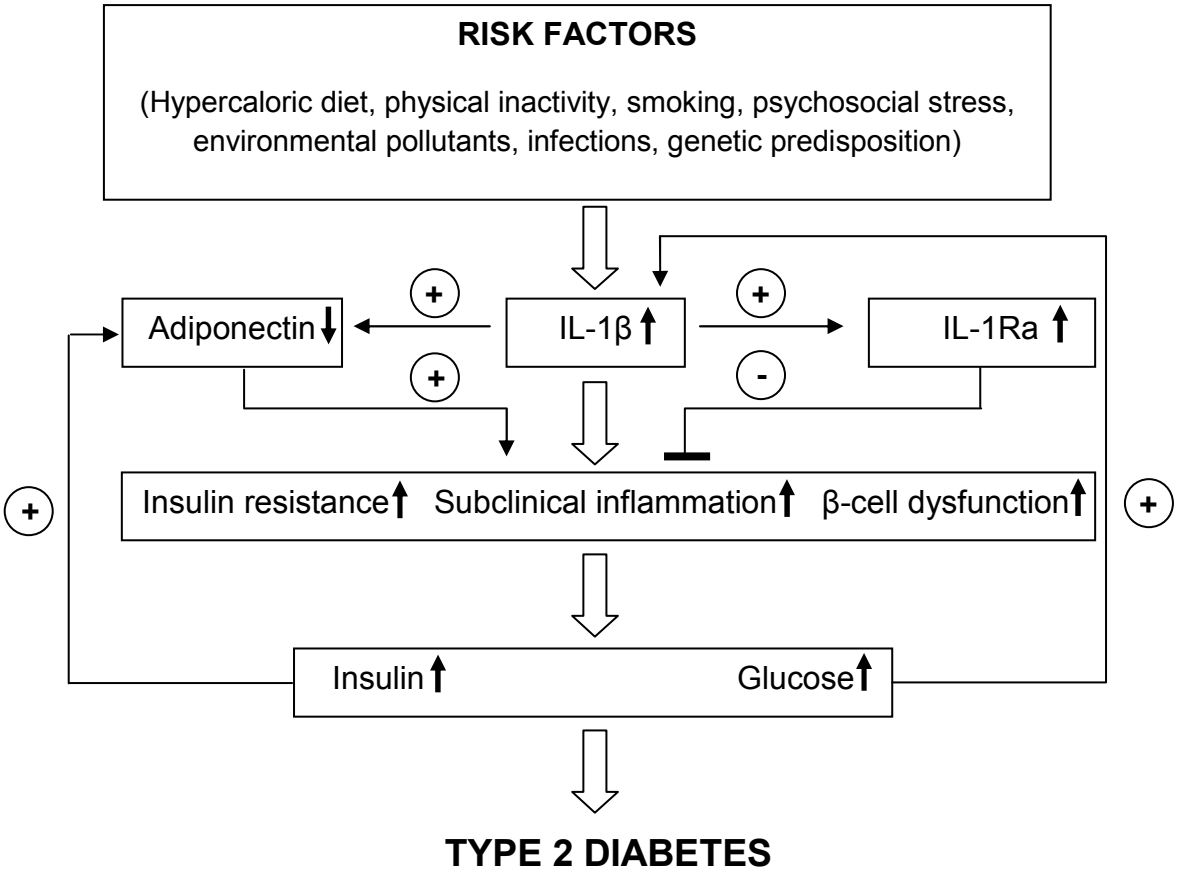

Figure 5: Model of the potential roles of IL-1Ra and adiponectin in the development of type 2 diabetes.

Risk factors such as unfavourable diet, physical inactivity, smoking, psychosocial stress, environmental pollutants and infections as well as genetic predisposition lead to an upregulation of the pro-inflammatory cytokine IL-1β. IL-1β in turn has the ability to promote insulin resistance, subclinical inflammation and β-cell dysfunction. In addition, IL-1β induces increased levels of its antagonist IL-1Ra and decreased levels of adiponectin. IL-1Ra tries to compensate the deleterious effects of IL-1β on β-cells and insulin-responsive tissues; an attempt that eventually fails. Decreased levels of adiponectin are associated with increased insulin resistance, β-cell dysfunction and subclinical inflammation. These inflammatory and metabolic disturbances result in an increase in insulin and glucose that in turn promote decreased levels of adiponectin and increased levels of IL-1Ra. In this hypothetical model, IL-1β represents one central mediator in the development of type 2 diabetes that initiates a fatal vicious circle eventually resulting in the development of type 2 diabetes (Fig. 5).

6.2 Macrophage inhibitory cytokine-1 (MIC-1) and the risk of incident type 2 diabetes

The main reasons for the investigation of the association between MIC-1 and the risk of incident type 2 diabetes were: (1) MIC-1 belongs to the TGF-β superfamily that also includes the anti-inflammatory TGF-β1 and (2) MIC-1 has anti-inflammatory properties and is inversely correlated with obesity similar to adiponectin.

Circulating levels of MIC-1 were determined in a prospective, nested case-control study within the Whitehall II study based on 180 individuals who subsequently developed type 2 diabetes during follow-up and 372 controls frequency-matched for age, sex and BMI who remained diabetes-free during follow-up.

The results of the experiments of our present study showed that circulating MIC-1 levels were significantly higher in individuals before type 2 diabetes manifestation compared to subjects who remained diabetes-free. This is contrary to our hypothesis that *increased* levels of MIC-1 are associated with a *decreased* risk of type 2 diabetes.

Confounders of the association between MIC-1 and incident type 2 diabetes

In our study, MIC-1 was significantly associated with incident type 2 diabetes with borderline significance after adjustment for age and sex. This association was attenuated after further adjustment for waist circumference, cardiovascular risk factors, socioeconomic status, pro-inflammatory mediators and glycaemia. This is in line with our finding of the association between baseline levels of MIC-1 and several variables, because MIC-1 showed numerous positive correlations with anthropometric factors (age, BMI, waist circumference and blood pressure), metabolic factors (total cholesterol, fasting triglycerides, fasting insulin and HOMA-IR) and immunological factors (CRP, IL-6 and IL-1Ra).

MIC-1 is regulated similarly to TGF-β1 rather than to adiponectin

The hypothesis that *higher* levels of MIC-1 are associated with a *reduced* risk of type 2 diabetes was based on its anti-inflammatory properties and its role in energy homoeostasis with increased levels being associated with weight loss;

similar to adiponectin. Higher levels of adiponectin were associated with a reduced risk of type 2 diabetes (110) and adiponectin is inversely associated with obesity (214). Based on the results of the present investigation, MIC-1 appears to be regulated in a different way than adiponectin. Increased levels of MIC-1 might rather reflect an attempt to counterbalance immunological and/or metabolic disturbances preceding type 2 diabetes as described above for IL-1Ra and as observed in a previous study on TGF-β1. The MONICA/KORA study demonstrated that elevated serum levels of TGF-β1 were associated with an increased risk of type 2 diabetes (114). In contrast to MIC-1, the association between TGF-β1 and incident type 2 diabetes was independent of anthropometric and metabolic factors. However, both immune factors, MIC-1 and TGF-β1 seem to be associated with other immune factors. Therefore, the association between increased levels of MIC-1 or TGF-β1 and an elevated risk of type 2 diabetes might be influenced by an interaction between different immune mediators and MIC-1 and TGF-β1, respectively. As MIC-1 belongs to the TGF-β superfamily, the coinciding results on increased levels of MIC-1 and TGF-β1 in association with an elevated risk of type 2 diabetes appear biologically plausible.

The role of MIC-1 in diabetes-related diseases

MIC-1 was also investigated in the context of cardiovascular events. In a prospective nested case-control study within the WHS based on over 27,000 healthy women, baseline levels of MIC-1 were higher in women who subsequently experienced cardiovascular events compared to those who remained event-free. In contrast to our results on MIC-1 and type 2 diabetes, this result was independent of classical cardiovascular risk factors (162). In a study with MIC-1-deficient mice it could be demonstrated that these mice died faster after a myocardial infarction than wild-type mice. This might be caused by damaged tissue that led to bleeding to death. The expression of MIC-1 seemed to be necessary to prevent this fatal complication (215). Therefore, MIC-1 has a protective role in cardiovascular events and further research is needed to demonstrate whether MIC-1 is also protective against type 2 diabetes, or mainly a counter-regulatory marker to reflect an upregulation of pro-inflammatory stimuli that affect the development of type 2 diabetes.

6.3 Secreted frizzled-related protein-5 (Sfrp5) in human serum

The main reason for the investigation of the characteristics of Sfrp5 in human serum was a recent report on similar properties of Sfrp5 and adiponectin in obese mice. Adiponectin and Sfrp5 were both primarily expressed in adipose tissue and their release was reduced in obesity. Both immune mediators are described as anti-inflammatory and capable of improving insulin sensitivity (145,163,216). As high levels of adiponectin are associated with reduced risk of type 2 diabetes (110), it can be postulated that Sfrp5 could also be protective against type 2 diabetes.

Circulating levels of Sfrp5 were investigated in a study based on 47 healthy study participants. The present data show that Sfrp5 was positively associated with HOMA-IR and oxidative stress. Sfrp5 showed no correlations with anthropometric and demographic data, apolipoproteins and subclinical inflammation.

This is in contrast to the results on adiponectin. Adiponectin was negatively associated with several indices of insulin resistance such as HOMA-IR and showed a positive correlation with apolipoprotein A-I, the main component of the atheroprotective high-density lipoprotein cholesterol. Therefore, the hypothesis that Sfrp5 is regulated in a similar way as adiponectin in humans could be refuted.

The role of Sfrp5 in the development of type 2 diabetes remains unclear

The present study design does not permit to evaluate whether or not Sfrp5 is protective against type 2 diabetes. Moreover, it is not possible to assess whether Sfrp5 is a cause or a consequence of insulin resistance and oxidative stress. So far, there are no further data on Sfrp5 in humans. It could be assumed that higher levels of Sfrp5 might be an attempt to counterregulate insulin resistance and oxidative stress as observed for IL-1Ra and MIC-1. However, it is also possible that Sfrp5 mediates oxidative stress and insulin resistance. Further analyses are on-going in our research group to clarify the direction of causality. The contradictory results on Sfrp5 from the present data and the data from Ouchi et al. (163) could be caused by differences in the investigated species (humans vs. mice) and/or by differences between the investigated tissue of Sfrp5 expression (blood vs. adipose tissue).

Sfrp5 has not yet been investigated in the context of other diabetes-related diseases such as cardiovascular events. Therefore, Sfrp5 is a novel immune mediator with potential species differences in its metabolic properties that needs to be characterised in more detail regarding its association with diabetes and with respect to its therapeutic potential as insulin-sensitising and anti-inflammatory protein.

6.4 Limitations and strengths

Limitations and strengths of the investigations on IL-1Ra, adiponectin and MIC-1 within the Whitehall cohort

There are two important *limitations* of the Whitehall cohort: (1) the study design for the investigation on the trajectories of IL-1Ra and adiponectin does not exclude alternative interpretations regarding the direction of causality. However, in the context of previous studies, assumptions on whether the results are a consequence or a cause of other outcomes can be made which need to be clarified by further studies. We were not able to measure levels of IL-1β and generate trajectories in a similar way as for IL-1Ra or adiponectin. This would have allowed a better interpretation of the relationship of IL-1Ra, IL-1β and adiponectin with each other and with incident type 2 diabetes. Moreover, we did not differentiate between the different molecular weights of adiponectin. Only total adiponectin levels were measured. Some studies reported that high molecular weight adiponectin might be more strongly associated with the development of type 2 diabetes (110,217,218), but there are also other studies that clearly indicate that measurement of adiponectin isoforms has no additional information compared to total adiponectin levels (203,219). Furthermore, we do not have data from all three study phases for other pro-inflammatory markers such as CRP and IL-6 to generate trajectories in the same way. This would allow a better interpretation of the interaction between pro- and anti-inflammatory immune mediators in the development of type 2 diabetes. (2) The Whitehall cohort is not population-based. The study participants worked in an office of the British Civil Service in the Whitehall Road London and white, male and middle-aged individuals were overrepresented. Therefore, our findings may not be generalisable.

The Whitehall cohort has four important *strengths*: (1) The Whitehall cohort is a well-phenotyped cohort, (2) most of the incident diabetes cases were diagnosed by the gold standard OGTT and (3) the present data on IL-1Ra and adiponectin are the first prospective investigations on the course of the levels of IL-1Ra and adiponectin based on up to three measurements from different time-points more than a decade before type 2 diabetes manifestation. (4) Moreover, the data on MIC-1 also represent the first prospective report on the association between MIC-1 levels and incident type 2 diabetes.

Limitations and strengths of the investigations on Sfrp5 within the Coffee Intervention Study

The most important *limitation* of the data on Sfrp5 is that the cohort is not population-based because the study participants are predominantly female and obese. Therefore, the data on Sfrp5 are not generalisable to lean and male subjects.

However, the cohort also has three notable *strengths*: (1) the study participants are well characterised with detailed information on anthropometric, demographic, metabolic and inflammatory parameters, (2) the OGTT as gold standard was used to assess glucose tolerance, and (3) the present report on Sfrp5 is the first one on this immune mediator in human serum.

6.5 Conclusion / Outlook

Individuals who subsequently develop type 2 diabetes are characterised by insulin resistance, β-cell dysfunction and hyperglycaemia more than a decade before diabetes diagnosis.

Subclinical inflammation is another mechanism contributing to the development of type 2 diabetes. It is characterised by chronically altered levels of expression and release of immune mediators whereby not only pro-inflammatory but also anti-inflammatory immune mediators are upregulated as shown for IL-1Ra and MIC-1. Higher levels of anti-inflammatory immune mediators obviously indicate a futile attempt to counterregulate the deleterious metabolic and inflammatory changes.

Adiponectin differs from other anti-inflammatory immune mediators because it is currently the only one which shows an inverse correlation with the risk to develop type 2 diabetes. However, its causal association is unclear because adiponectin seems to be linked to insulin action in a bidirectional way. On the one hand, decreased levels of adiponectin might be a cause of impaired insulin sensitivity because adiponectin was found to have insulin-sensitising characteristics. On the other hand, reduced levels of adiponectin might be a consequence of deteriorated insulin sensitivity because adiponectin concentrations are suppressed by increased levels of insulin in insulin-resistant individuals.

The investigation of anti-inflammatory immune mediators is clinically relevant because they might represent interesting novel therapeutic targets. Recent data showed that the anti-inflammatory Sfrp5 shares several features with the diabetes-protective adiponectin in obese mice. However, our data demonstrate that Sfrp5 levels in human blood are regulated differently than adiponectin levels. Discrepancies between our study and previous investigations in mice point to species and tissue-specific differences in Sfrp5 regulation. Therefore, it could not be excluded that Sfrp5 has anti-inflammatory and insulin-sensitising characteristics in other tissues such as adipose tissue as described in mice. More mechanistic are on-going to clarify these questions.

Further investigations on the trajectories of other anti- and pro-inflammatory immune mediators are planned as they should lead to a better understanding of the role of subclinical inflammation before type 2 diabetes manifestation and might

help to disentangle the issue of cause and consequence regarding metabolic disturbances in more detail. We are also planning the comparison with trajectories of other biomarkers (glucose metabolism, biomarkers of non-alcoholic fatty liver disease, biomarkers of endothelial dysfunction) in order to better understand the temporal order of pathophysiological changes in the development of type 2 diabetes.

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* contributed equally to this study

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Carstensen M, Herder C, Kivimäki M, Jokela M, Roden M, Shipley M, Witte DR, Brunner EJ, Tabák AG.
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45th Annual Meeting of the European Association for the Study of Diabetes, Vienna (Austria); 29 Sep – 02 Oct 2009. **Oral presentation**

Carstensen M, Herder C, Brunner EJ, Strassburger K, Tabák AG, Roden M, Witte DR.
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"Adiponectin trajectories preceding the development of type 2 diabetes mellitus - 13 year observation in the Whitehall II study"
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„Assoziation zwischen der genomweiten Genexpression im humanen Vollblut und Nüchtern- sowie 2-Stunden-Glukose: KORA F4 Studie"
46th Annual Meeting of the German Diabetes Association, Leipzig (Germany); 01 - 04 June 2011. **Poster presentation**

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"The adipokine Sfrp5 is associated with oxidative stress and is regulated by glucose"
6th Annual Meeting of the Central European Diabetes Association, Zurich (Switzerland); 30 June – 2 July 2011. **Poster presentation**

Carstensen M.
"Anti-inflammatory cytokines and risk of type 2 diabetes: data from epidemiological cohort studies".
16th Hagedorn-EASD Oxford Workshop, Oxford (UK); 05 – 08 Aug 2011. **Invited oral presentation**

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"Genome-wide gene expression in peripheral blood of type 2 diabetes patients and subjects with normal glucose tolerance: the population-based KORA Survey F4"
47th Annual Meeting of the European Association for the Study of Diabetes, Lisbon (Portugal); 12 - 16 Sep 2011. **Poster presentation**

Carstensen M.
"Interleukin-1 receptor antagonist and adiponectin trajectories preceding the diagnosis of type 2 diabetes mellitus – Whitehall II prospective study"
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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Düsseldorf, 01. Februar 2012 Maren Carstensen