Clinical and Pathophysiological Aspects of Type 2 Diabetes In South Asian Immigrants to Norway

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To my patients

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Abbreviations

AIRg: acute insulin response to glucose

ALAT: alanine amino-transferase

AMPK: adenosine monophosphate-kinase

ANCOVA: analysis of covariance

Anti-GAD: anti-glutamic acid decarboxylase

Anti-IA2: autoantibodies to Protein Tyrosine Phosphatase

ASAT: aspartate amino-transferase

ATP: adenosine triphosphate

ATP III: Adult treatment panel III

AUC: area under the curve

BIA: bioelectrical impedance analysis

BMI: body mass index

CNS: central nervous system

CRP: C-reactive protein

CT: computed tomography

CVD: cardiovascular disease

DAG: diacyl-glycerol

DELFIA: Dissociation Enhanced Lanthanide Fluoro-immunoassay

DIPI: Diabetes in Pakistani immigrants

DIVINE: Diabetes Intervention trial with Vitamin D in subjects of sub-Indian and Nordic Ethnicity

DSAT: deep subcutaneous adipose tissue

DXA: dual X-ray absorptiometry

EGP: endogenous glucose production

ELISA: Enzyme linked immunosorbent assay

EVF: erythrocyte volume fraction

FFM: fat-free mass

GCP: good clinical practice

GIR: glucose infusion rate

GLUT: glucose transporter

IκB-α: inhibitor of kappa B-alpha

GWAS: genome wide associations' studies

HbA_{1c}: Haemoglobin A_{1c}

HDL: high-density lipoprotein

HMW: high molecular weight

HOMA: homeostasis model assessment

HU: Hounsfield units

IC: Indirect calorimetry

IDF: International Diabetes Federation

IKK: Inhibitor of kappa B kinase

IL-1β: Interleukin-1 beta

IL-1R: Interleukin-1 receptor

IL-1RA: Interleukin-1 receptor agonist

IL-6: Interleukin-6

IL-10: Interleukin-10

IMAT: inter-muscular adipose tissue

IQR: inter-quartile range

IR: insulin receptor

IRS: Insulin receptor substrate

ISI: Insulin sensitivity index

IVGTT: intra-venous glucose tolerance test

JNK: JUN N-terminal kinase

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LDL: low-density lipoprotein

LS-ratio: liver-spleen ratio

MAP: mitogen activated protein

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

NEFA: Non-esterified fatty acids

NF-κB: nuclear factor kappa B

NLRP3: Nod-like receptor protein 3

OMB: Office of Management and Budget

P38MAPK: p38 MAP kinase

PI3K: phosphatidylinositol 3-kinase

PKB: Protein kinase B

PKC: Protein kinase C

PKR: double stranded RNA-dependent protein kinase

PPAR: peroxisome proliferator-activated receptor

PTP1B: protein-tyrosine phosphatase 1B

RIA: radio-immuno assay

ROI: region of interest

RQ: respiratory quotient

SAT: subcutaneous adipose tissue

SFAT: sub-fascial adipose tissue

SSAT: Superficial subcutaneous adipose tissue

TGD: total glucose disposal

TLR: toll like receptor

TNFα: tumour necrosis factor alpha

TSAT: thigh subcutaneous adipose tissue

UCP: uncoupling protein

UV: ultra-violet

VAT: visceral adipose tissue

WHO: World Health Organization

WHR: waist-hip ratio

List of papers

Paper I

Wium C, Aasheim ET, Ueland T, Michelsen A, Thorsby PM, Larsen IF, Torjesen PA, Aukrust P, Birkeland KI. **Differences in insulin sensitivity, lipid metabolism and inflammation between young adult Pakistani and Norwegian patients with type 2 diabetes: a cross sectional study.** *BMC Endocrine Disorders* 2013 **13**:49.

Paper II

Wium C, Eggesbø HB, Ueland T, Michelsen AE, Torjesen PA, Aukrust P, Birkeland KI. **Adipose tissue distribution in relation to insulin sensitivity and inflammation in Pakistani and Norwegian subjects with type 2 diabetes** *Submitted*

Paper III

Wium C*, Gulseth HL*, Eriksen EF, Birkeland KI. **Characteristics of glucose metabolism in Nordic and South Asian subjects with type 2 diabetes.** *PLoS One* 2013 **8**(12):e83983.

1 Introduction

1.1 Type 2 diabetes

"The term "diabetes mellitus" describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs" (World Health Organization 1999).

Type 2 diabetes is the most common form of diabetes, constituting 90 % of the estimated 382 million diabetes cases worldwide in 2013 (1). It was for a long time known as adult-onset diabetes, but in recent years, with increasing prevalence of obesity, also adolescents and even children are developing type 2 diabetes, particularly in some ethnic groups (2;3). Type 2 diabetes is becoming a serious global health problem, especially in countries of low and middle income, where the prevalence is increasing the most, and where the age of debut is relatively low. The World Health Organization (WHO) estimates that diabetes will be the 7th most common cause of death by 2030 (4).

The pathogenesis of type 2 diabetes is not fully understood. It is a disease, or probably several diseases of multifactorial aetiology. However, we know that development of type 2 diabetes requires two major elements: insulin resistance, leading to ineffective insulin action, and β-cell dysfunction, leading to inappropriate insulin secretion. In this introduction, I will present a brief overview of mechanisms known to be involved in the pathogenesis of type 2 diabetes. I will also discuss the concept of ethnicity and describe elements that are characteristic of type 2 diabetes in South Asians, both in their countries of origin and as immigrants to Western countries.

1.1.1 Insulin resistance

Insulin resistance can be described as a reduced biological response to insulin, primarily to its acute effects on glucose and lipid metabolism. This is pertinent as whether the insulin is produced endogenously or administered exogenously, eventually leading to hyperglycaemia. Insulin resistance occurs in several tissues, mainly as decreased insulin dependent glucose

uptake in skeletal muscle, decreased ability of insulin to inhibit lipolysis in adipose tissue, impaired insulin mediated inhibition of endogenous glucose production in liver (5) and decreased insulin enhanced glucose-induced insulin secretion in pancreas (6). The causes and mechanisms of insulin resistance are still unclear, but it is obvious that both type 2 diabetes and insulin resistance are closely related to obesity.

Insulin resistance is also present in several other conditions not directly related to type 2 diabetes or the metabolic syndrome, both physiological (pregnancy, puberty, stress, old age) and pathological (illness, starvation, uraemia, liver cirrhosis) (7).

Insulin sensitivity is a term that is also often used in literature, and it is the reciprocal of insulin resistance. Normal limits of insulin sensitivity are wide, often overlapping with some pathological conditions. It gives more sense to talk about “normal insulin sensitivity” than “normal insulin resistance”.

NEFA

Non-esterified fatty acids (NEFA) have been shown to play an important role in insulin resistance, both in skeletal muscle and in liver (8), and have been suggested as one of several possible mechanisms for the development of insulin resistance. NEFA seem to inhibit skeletal muscle intracellular insulin signalling pathways, by inhibiting tyrosine phosphorylation of insulin receptor substrates (IRS-1 and -2), and IRS-1 and-2 associated phosphatidylinositol 3-kinase (PI3K) activity *(Figure 1)*. High plasma NEFA concentrations induce a substantial increase in intracellular NEFA and its metabolites, such as ceramide and diacyl-glycerol (DAG). These in turn activate protein kinase C (PKC) isoforms, again activating other kinases like the JUN N-terminal kinase (JNK) and inhibitor of κB kinase (IKK). JNK and IKK, through phosphorylation of serine/threonine residues on the insulin receptor and its substrates, inhibit insulin signal transduction, thus inhibiting the kinase Akt/protein kinase B (PKB) activity. Inhibiting Akt/PKB results in decreased glucose transport by reducing recruitment of GLUT4 transporters to the cell membrane, reduced glycogen synthesis, reduced suppression of glycogenolysis in liver, reduced protein synthesis and reduced inhibition of lipolysis *(Figure 1)* (5). IKK also phosphorylates the inhibitor of kappa B-alpha (IκB-α), an inhibitor of nuclear factor kappa B (NF-κB). This leads to dissociation of IκB-α from NF-κB, and thus NF-κB is free to migrate into the nucleus and promote transcription of proinflammatory cytokines (9), as will be viewed in the section on inflammation.

Figure 1: Insulin signalling pathways

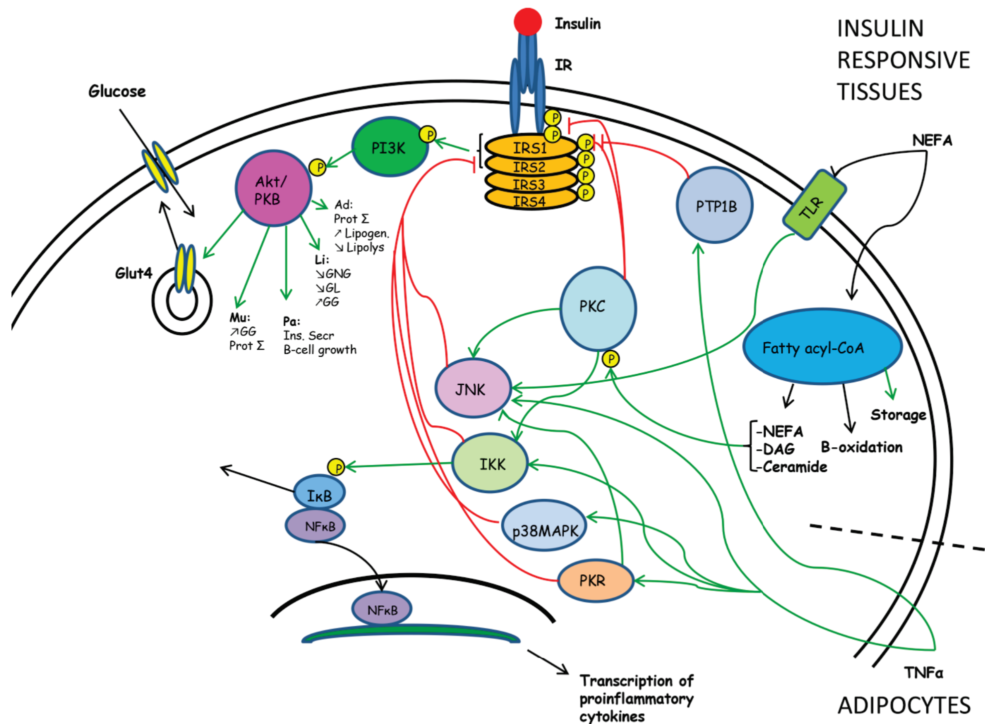


Figure 1: Simplified representation of intracellular insulin signalling in insulin responsive tissues, and effect of non-esterified fatty acids (NEFA) and tumour necrosis factor alpha (TNFα) (in adipocytes). Green arrows represent activation, red links represent inhibition. For further explanations, see text. IR: insulin receptor, IRS: insulin receptor substrate, TLR: toll-like receptor, PI3K: phosphatidylinositol-3-kinase, Akt/PKB: Akt/protein kinase B, Glut4: glucose transporter 4, DAG: diacyl-glycerol, PKC: protein kinase-C, JNK: JUN N-terminal kinase, IKK: inhibitor of kappa B-kinase, p38MAPK: p38 MAP-kinase, PKR: double stranded RNA-dependent protein kinase, IκB: inhibitor of kappa B, NFκB: nuclear factor kappa B, PTP1B: protein-tyrosine phosphatase 1B. P: phosphorylation. Ad: adipose tissue, Li: liver, Pa: pancreas, Mu: muscle, GG: glycogenesis, Prot Σ: protein synthesis, Ins. Secr.: insulin secretion, GNG: gluconeogenesis, GL: glycogenolysis, Lipogen: lipogenesis, Lipolys: lipolysis. (Wium C. 2013).

Inflammation

Adipose tissue and the immune system have common ancestry, both originating from the mesoderm, and in more primitive organisms, like the Drosophila, they are one single organ,

the fat body (10). NF-κB is a group of transcription factors, which plays a pivotal role in inflammation. Obesity results in low grade chronic inflammation, by some authors termed "metaflammation" (11). Overweight and obese adipose tissue secretes inflammatory markers and cytokines/adipokines such as leptin, adiponectin, C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), which have been closely linked to insulin resistance. Inflammation increases the infiltration of immune cells, mainly macrophages, but also lymphocytes and mast cells, into the adipose tissue, in turn contributing to the increasing secretion of cytokines (11).

Macrophages are probably the main source of adipose tissue TNF-α production (12). TNF-α acts in many of the same ways as NEFA, inhibiting the insulin receptor and the intracellular signalling pathways through activation of IKK (5). The IKK complex is an important serine/threonine kinase, which inhibits the insulin receptor IRS-1 through phosphorylation of serine residues, thereby inducing insulin resistance. It also inhibits the IκB-α through phosphorylation, so that NF-κB becomes free to move into the nucleus and activate inflammatory target genes. TNF-α in addition inhibits tyrosine phosphorylation of the insulin receptor and IRS-1 through activation of protein-tyrosine phosphatase 1B (PTP1B) (5), and activates other intracellular serine kinases, like p38 MAP kinase (p38MAPK), JNK and double stranded RNA-dependent protein kinase (PKR) (11) *(Figure 1)*. PKR is important in pathogen sensing, but is also activated by obesity or infusion of lipids. It stimulates JNK and inhibits IRS through serine phosphorylation (13).

Whereas TNF-α mainly acts locally, in a paracrine manner, IL-6 is a pleiotropic, endocrine cytokine, with target cells far away from its site of production. IL-6 is produced in a number of cells, including both macrophages and adipocytes, and is regarded as both a proinflammatory and anti-inflammatory cytokine, with effects in many tissues (14). Adipose tissue contributes approximately 15-35% of basal circulating IL-6 (15), with visceral adipose tissue producing three times more IL-6 than subcutaneous adipose tissue in obese subjects (16). It is estimated that10-12 % of this production comes from adipocytes (17). IL-6 is the most important circulating factor controlling the acute phase response of the liver (including production of CRP, serum Amyloid A, Haptoglobin and others) (18). In the bone marrow, IL-6 increases platelet production. In vascular endothelium it increases platelet activity, expression of adhesion molecules and activates local renin-angiotensin, leading to inflammation and damage of the vessel wall (19). In the central nervous system (CNS),

secretion of IL-6 from neurones and glial cells, among other effects induces a strong catabolic reaction, and also causes reduced food intake and increased energy expenditure (19). IL-6 has been shown to be associated with both obesity and type 2 diabetes (20).

CRP is an acute phase reactant that is present in high concentrations in the circulation. It is mainly produced by the liver, but also secreted by non-fat cells (immune cells) of the adipose tissue (14). CRP has been shown to be increased in obesity (21) and type 2 diabetes (20).

Leptin is a hormone secreted by adipocytes, with an important role in mediating energy balance. It acts as a signal to the brain on the status of lipid stores (14). Leptin production is higher in subcutaneous than in visceral fat (22). Although the adipocyte is the main site of leptin production, low levels of expression have also been detected in placenta, skeletal muscle, mammary epithelium and brain (23). When leptin is reduced during fasting, the hypothalamus will stimulate appetite and reduce energy expenditure. During famine leptin also causes reduction in thyroid hormone production and the reproductive hormonal axis is inhibited (24). The immune system is also inhibited (15% of daily energy expenditure is spent by the immune system), through direct regulation of T lymphocytes and cytokine production (25). When Friedmann and colleagues discovered leptin in 1994, there were initially high hopes for a therapeutic effect of leptin on obesity. However, in most subjects with obesity, leptin levels are high, and treatment with leptin has not been successful in producing weight loss, possibly due to central leptin resistance in obesity (26). Leptin is partly regulated by insulin. Low insulin levels decreases leptin, while leptin increases with feeding or in response to insulin stimulation (23). Leptin activates AMP-kinase (AMPK) in peripheral tissues like muscle, leading to increased fatty acid oxidation. This might be a reason for leptin's ability to improve insulin resistance in subjects with leptin deficiency (27). Leptin also activates other signalling pathways like MAPK and PI3K in insulin sensitive tissues, overlapping with insulin signalling pathways, which may be a mechanism for leptin's insulin sensitizing effects, and may suggest a common pathogenesis of leptin resistance and insulin resistance (28).

Adiponectin is an anti-inflammatory signal substance mainly produced in adipose tissue, but may also be produced in several other tissues, such as skeletal myocytes, cardio-myocytes and endothelium (29). Plasma concentration is high and correlates inversely to white adipose tissue, especially visceral adipose tissue. Adiponectin is also strongly correlated with insulin sensitivity, with lower adiponectin levels in subjects with type 2 diabetes than in controls,

even when matched for BMI (30). Adiponectin exists in the circulation in several isoforms, such as trimers, hexamers and multimers of high molecular weight (HMW), where the HMW-adiponectin is purportedly the most active (31). There is also a globular form, with only the C-terminal domain, often trimerised, but lacking the collagen-like side chains. There are two main adiponectin receptors, the AdipoR1, AdipoR2. In addition, T-cadherin can also function as a receptor for medium and high molecular weight adiponectin (29). It has been shown in mouse and human cell cultures that when adiponectin binds to its receptor, it can activate the intracellular AMPK signalling cascade, as well as peroxisome proliferator-activated receptor (PPAR) α and p38MAPK. In the liver, activation of PPARα in addition to AMPK leads to increased fatty acid oxidation and inhibition of fatty acid synthesis (32). Globular adiponectin binding to AdipoR1 has been shown to suppress TLR-induced NF-κB activation in mouse macrophages (33). Adiponectin also increases the expression of anti-inflammatory cytokines like the IL-10 and Interleukin-1 receptor agonist (IL-1RA) in human macrophages (34). However, in humans, some authors argue that the relationship between adiponectin and insulin action is more complex than what has initially been proposed in light of animal studies. Adiponectin is often regarded as an insulin sensitizing hormone, whereas some argue that currently available evidence rather suggests a role of adiponectin as a downstream signal resulting from hyperinsulinaemia, secondary to insulin resistance, and not causing it (31). This would also explain the higher levels of adiponectin seen in type 1 diabetes.

The IL-1 cytokine family is large, and plays a central role in immune and inflammatory responses. IL-1 consists of two major proteins, IL-1α and IL-1β, encoded by different genes, but structurally related, such that they bind to the same receptors, IL-1RI and IL-1RII. Binding to IL-1RI induces a potent proinflammatory reaction, with various biological and metabolic effects, inducing insulin resistance (35). IL-1RII probably acts as a suppressor of IL-1 activity by competing for IL-1 binding, acting as a decoy (36). IL-1β maturation is regulated by a multi-protein complex called the Nod-like receptor protein 3 (NLRP3) inflammasome (37). The IL-1RA also binds to the receptor IL-1RI, inhibiting the intracellular response (38). IL-1RA is considered an anti-inflammatory cytokine, that antagonises IL-1β and IL-1α, and is elevated, at least in part, in response to elevation of these inflammatory cytokines (17). An early study has showed decreased IL-1RA levels in type 2 diabetes (39), and it has been demonstrated that leptin decreases β-cell production of IL-1RA, down-regulating IL-1RA expression in pancreatic β-cells in type 2 diabetes (40). However, studies that are more recent have shown high levels of IL-1RA in obesity, correlating with BMI,

insulin resistance and serum leptin levels. Increased production of IL-1RA in adipose tissue has been shown in obese humans (41-43). Subjects with impaired glucose tolerance have higher levels of IL-1RA (44), and in two prospective cohort studies, IL-1RA was found to be elevated several years before diabetes diagnosis, and significantly predicted incident diabetes (45;46). Whether this represents a counteracting mechanism in response to IL-1 is at present unclear. IL-1 circulates at much lower levels in plasma and is more difficult to detect in clinical samples.

1.1.2 β-cell dysfunction

β-cell dysfunction is a crucial factor in the development of type 2 diabetes. Although serum insulin and C-peptide levels can be substantially higher in subjects with type 2 diabetes than in healthy subjects, insulin secretion in type 2 diabetes is inadequate to the body's needs, and thus the β-cells are unable to adequately compensate for the increased insulin demand. β-cell dysfunction has therefore been recognized as a fundamental component in type 2 diabetes (47). It is noteworthy that most of the genetic variants linked to type 2 diabetes actually concern genes active in β-cells and/or involved in insulin secretion, and only a few are involved the processes known to be related to insulin resistance (48). Recent publications, however, implicate insulin resistance at the level of the β-cell in the development of β-cell dysfunction (6). The β-cell is in fact insulin responsive, with insulin enhancing the glucose-induced insulin secretion in healthy humans, and this independently of changes in NEFA (49;50). In insulin resistant subjects this effect has been shown to be reduced (6).

1.1.3 Glucose and lipid metabolism

Fasting state

In the fasting (post-absorptive) state, endogenous glucose production maintains euglycaemia, which is important for the function of several tissues using glucose as their sole source of energy. This includes the brain and red blood cells (51). The liver produces approximately 80 % of endogenous glucose during the post-absorptive state, through glycogenolysis and gluconeogenesis. The liver stores approximately 80 grams of glycogen before an overnight fast (52). Glycogen reserves can then be degraded into glucose through glycogenolysis. The liver also produces glucose through gluconeogenesis, from substrates either issuing from non-

oxidative glucose degradation, (lactate and pyruvate) or from lipid degradation (glycerol) *(Figure 2)*. Certain amino acids (alanine) can also be used as substrate (53). The remaining 20 % of endogenous glucose is produced mainly by gluconeogenesis in the kidneys (51). In the post-absorptive state, the body uses lipids as its main source of energy, through fatty acid β-oxidation. However, approximately 25 % of resting energy expenditure still uses carbohydrates, where the brain and erythrocytes are responsible for 50 % of glucose consumption (54).

In the post-absorptive state, insulin secretion is low due to normal/low levels of plasma glucose. Glucagon and other gluco-regulatory hormones, like catecholamines, growth hormone, cortisol and thyroid hormone, act to increase endogenous glucose production. Glucagon increases glycogenolysis and gluconeogenesis in the liver, whereas adrenalin mainly increases renal gluconeogenesis. Growth hormone, cortisol and thyroid hormone have a more indirect mode of action, with a slower effect, modulating the effects of insulin on tissues (51).

Figure 2: Glucose metabolism in liver

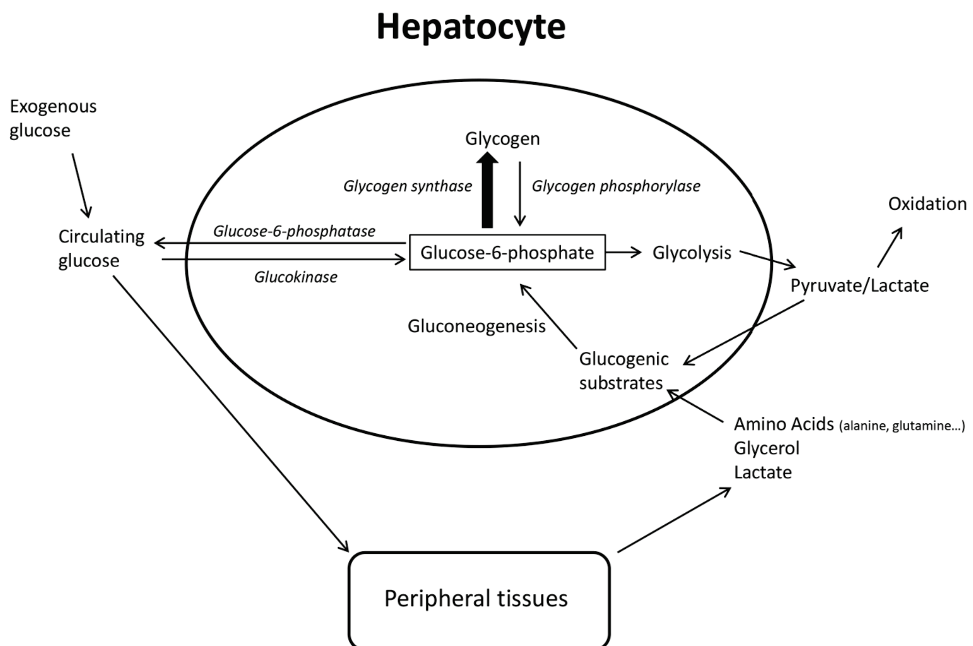


Figure 2: Glucose metabolism in liver. Adapted from Radziuk J et al. (53)

Postprandial state

In the post prandial state, glucose and insulin levels rise, and glucagon levels are reciprocally suppressed (51). Insulin facilitates glucose uptake into insulin sensitive tissues like muscle and adipose tissue, through increased translocation of the insulin dependent GLUT 4 transporters to the cell membrane *(Figure 1)* (55).

In the muscle, insulin inhibits proteolysis and lipolysis, and stimulates glycolysis via hexokinase and phosphofructokinase phosphorylation (55). In muscle resting conditions, approximately 30 % of the glucose load is removed from the circulation by muscle (51). In the muscle, glucose is quickly phosphorylated into glucose-6-phospate, and either broken down into tricarboxylic compounds used in the citric acid cycle in the mitochondria for energy production (oxidation - less than10 % of the total glucose load), or stored as glycogen for later use (less than 20 % of the total glucose load). Insulin activates muscle glycogen synthase, stimulating glycogen storage. Muscle glycogen stores are limited, however. After repletion of glycogen stores, excess glucose is metabolised through glycolysis into pyruvate, alanine and lactate, and then transported back to the liver to be used later as substrate in gluconeogenesis and glycogen synthesis. This is termed the Cori cycle/lactic acid cycle, or the glucose-alanine cycle *(Figure 3)* (52).

In adipose tissue, insulin also increases glucose uptake through increased GLUT 4 translocation. Lipolysis and liberation of NEFA is inhibited, and fatty acid and triacylglycerol synthesis is stimulated. Glycolysis is stimulated in the adipose tissue via hexokinase and phosphofructokinase phosphorylation, as in muscle. Glucose is then either used as energy substrate or metabolised into pyruvate, alanine or lactate which can be released into the circulation and transported back to the liver for gluconeogenesis and glycogen synthesis (the Cori cycle) (55).

In the liver, the GLUT 2 transporters are not insulin dependent, but are sensitive to plasma hyperglycaemia. So is the hepatic glucokinase that phosphorylates glucose into glucose-6-phosphate inside the hepatocytes. The further steps of polymerisation of glucose for storage as glycogen are however hormone dependent. Insulin stimulates glycogen synthase and, together with hyperglycaemia, strongly inhibits glycogen phosphorylase *(Figure 2)*. Glucagon and adrenaline have an opposite effect to insulin (51). Insulin inhibits gluconeogenesis through inhibition of key enzymes in the process. Insulin also inhibits the use of lipids as energy

substrate, by activating triglyceride synthesis and by favouring glucose as energy substrate instead of NEFA.

Figure 3: Glucose delivery and metabolism.

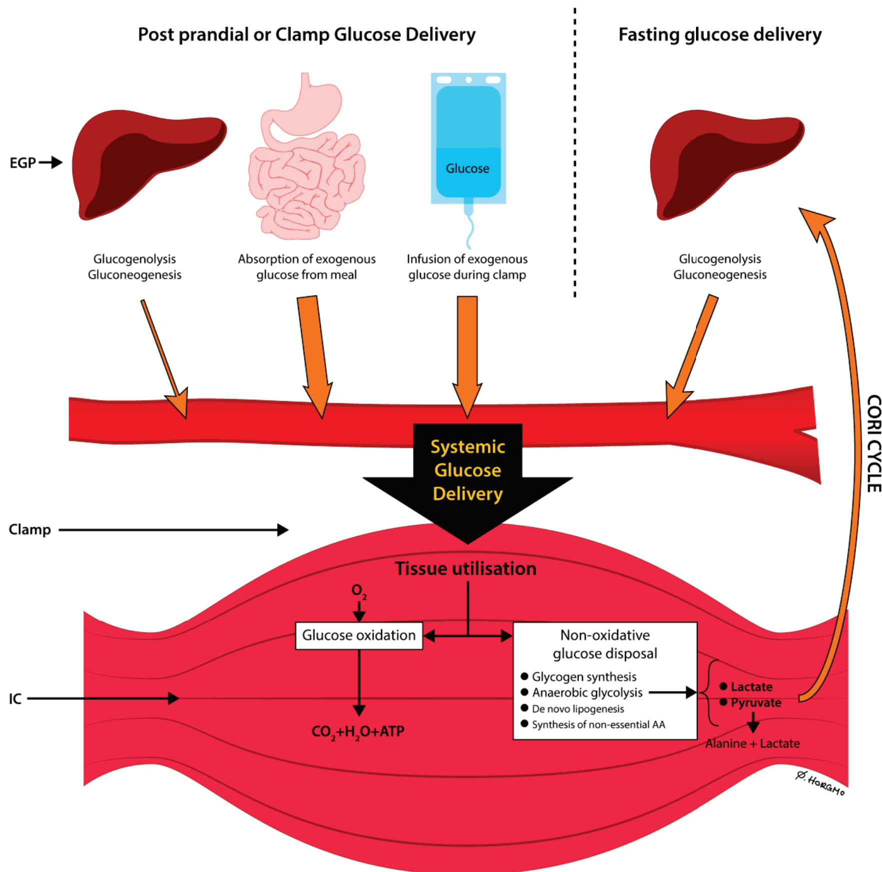


Figure 3: Glucose delivery and metabolism in the fasting, post-absorptive state, in the post-prandial state and during hyperinsulinaemic clamp. In the post-absorptive state circulating glucose levels are maintained by hepatic endogenous glucose production (EGP) through glycogenolysis and gluconeogenesis. In peripheral tissues, glucose is oxidized for energy production, or disposed of through non-oxidative metabolism, mainly anaerobic glycolysis, resulting in pyruvate, lactate and alanine, which are transported back to the liver through the Cori cycle or the glucose-alanine cycle and used as substrates for gluconeogenesis. In the post-prandial or clamp state the endogenous glucose production is decreased or suppressed, and exogenous glucose from gut absorption or intravenous infusion is the main source of circulating glucose. IC: indirect calorimetry. (Wium C. 2013).

During clamp hyperinsulinaemia

During the hyperinsulinaemic, euglycaemic clamp, the high physiologic or supra-physiologic levels of insulin will have the same effects on the insulin sensitive tissues as in the post-prandial state previously described. Glucose uptake into muscle is promoted, as well as inhibition of hepatic glucose production, through inhibition of both glycogenolysis and gluconeogenesis. Inhibition of lipolysis and NEFA liberation from adipose tissue is also seen. In healthy subjects with normal insulin sensitivity, clamp hyperinsulinaemia is thought to inhibit hepatic glucose production almost entirely, although this is also debated. In subjects with type 2 diabetes, with varying levels of insulin resistance, hepatic glucose production is variably, and only partly inhibited (56).

1.1.4 Body composition and adipose tissue compartments

Anthropometry

Measuring the body size and composition is important for the evaluation of a person's risk of metabolic disease. Measurements of body height and weight are simple to carry out, and can be used to calculate the body mass index (BMI) = $\frac{weight\ (in\ kg)}{height\ (in\ m)^2}$, which is widely used to diagnose underweight, overweight and obesity, both in research and in clinical practice.

The WHO has defined BMI cut off points, with underweight BMI < 18.5 kg/m^2, normal BMI 18.5 – 24.9 kg/m^2, overweight as BMI 25.0 – 29.9 kg/m^2, and obese as BMI > 30.0 kg/m^2, with obese I as BMI 30.0 – 34.9 kg/m^2, obese II as BMI 35.0 – 39.9 kg/m^2, and obese III as BMI > 40.0 kg/m^2 (57).

There are ethnic differences in the relationship between BMI values and health risks, where Asian and Pacific populations have cut-off points for overweight varying between 22 to 25 kg/m^2. Therefore, the WHO Expert Consultation in 2004 recommended to keep the current cut-off points, but adding additional cut-off points at 23.0, 27.5, 32.5 and 37.5 kg/m^2 for public health actions (58). An Indian consensus statement from 2009 presented revised guidelines for the diagnosis of obesity, abdominal obesity and the metabolic syndrome in Asian Indians (59). They used the following limits of BMI: normal weight: 18.0 – 22.9, overweight: 23.0 – 24.9, obesity: ≥ 25.0.

Concerning abdominal obesity, the WHO is currently further reviewing available data on the relation between waist circumference and morbidity (57). Measurement of waist and hip circumference, and the calculation of the waist to hip ratio, and the waist to height ratio have often been used, and have shown variable relations to metabolic disease and mortality (60).

The Indian consensus statement from 2009 defined waist circumference cut-offs for obesity lower than in the ATP III guidelines (61): in men ≥ 90 cm vs. ≥ 102 cm, and in women: ≥ 80 cm vs. ≥ 88 cm (62). These new cut-offs were in agreement with the IDF consensus worldwide definition of the metabolic syndrome (63) and the “Harmonizing the Metabolic Syndrome” joint interim statement from the IDF and several other heart, lung and obesity organisations (64).

Bioelectrical impedance analysis

For more direct measurement of fat mass and fat-free mass, the bioelectrical impedance analysis is a simple method for assessing body composition, readily available in an outpatient clinic, with affordable equipment. BIA measures total body water from given height, measured weight and measured impedance, or opposition, to the flow of an electrical current sent through the body. It uses built-in software equations for the estimation of fat mass and fat free mass in whole body, extremities and trunk (65). There is a variability in hydration and density of fat free mass in people of different age, sex and ethnicity, implying a need for using equations that have been developed specifically for the population to be studied (65). So far, BIA equations have been validated mainly on white populations, although some validated equations for other populations exist (66-68). Validations are usually performed against isotope dilution (double-labelled water) measurements of body composition or dual energy X-ray absorptiometry (DXA).

Dual energy X-ray absorptiometry

DXA is a method for measuring total body composition, as well as bone mineral density. Two x-ray beams with different energy levels are sent through the whole body, and calculation of body composition from differences in absorption is made (65). DXA has the obvious advantage of giving estimations of body composition in total and segmental parts of the body while exposing the subject to minor amounts of radiation (69). It is more time-consuming than BIA, and requires more expensive equipment, but is still relatively easy to use, with low

operational cost, and is much used in both clinical practice and research. A disadvantage to DXA, and to BIA, is that they are not capable of discriminating between different abdominal adipose tissue compartments. DXA measurements generally have good precision. However, the variation in measurements using DXA devices from different manufacturers is large (69).

Computed tomography

Computed tomography (CT) scans are also used to measure body composition. CT measurements have the advantage of giving rapid and accurate analyses of body composition, where the different tissue compartments can be assessed (65). Analyses of CT scans are based on differences in attenuation between various tissues and compartments. Water has an attenuation of zero on the Hounsfield scale, whereas air is minus 1000 Hounsfield units (HU) and bone is + 400 to + 2000 HU. Muscle has an attenuation of + 10 to + 40 HU, liver is + 40 to + 60 HU and fat is usually – 100 to – 50 HU. CT is thus capable of discerning different tissue compartments, such as adipose tissue compartments, muscle compartments etc., but also to assess the degree of ectopic lipid infiltration in tissues, such as the liver or skeletal muscle (70;71). A disadvantage with CT is the exposure to radiation with whole body measurements (69). A limited number of slices are therefore often performed, providing information on regional body composition.

Liver

When measuring the liver with CT scan, the interest is upon the degree of fatty infiltration. This can be assessed by measuring the liver attenuation in unenhanced CT scans, in designated regions of interest (ROI). Lower attenuation, signifies higher fat content in the liver. The liver attenuation is often compared to the attenuation in the spleen, which is not prone to lipid infiltration, and normally has an attenuation a little lower than the liver (around + 50 HU). The liver-spleen ratio (LS-ratio) is normally about 1.2 (72). Measurements using three ROI in the liver and two ROI in the spleen of one single slice through the liver at the abdominal level *(Figure 4A)* is said to be optimal and give reproducible results (73).

Subcutaneous abdominal adipose tissue

A CT slice through the mid-abdomen is useful for assessment of the various abdominal adipose tissue compartments *(Figure 4B)*. The subcutaneous adipose tissue can be sub-divided into superficial (SSAT) and deep subcutaneous adipose tissue (DSAT) by the

superficial fascial plane (74), and studies indicate that there are differences in both the structure and function of these two subcutaneous compartments, with DSAT being more closely related to insulin resistance, like visceral adipose tissue (VAT) (75). Abdominal adipose tissue area on the CT slices will vary according to level of measurement. Choosing the best level to measure must take into account the subject's sex, due to differences between male and female anatomy. Shen et al. proposes optimal measuring levels at 5 cm above L4-L5 in women and 10 to 15 cm above L4-L5 in men (76).

Figure 4: CT measurements of adipose tissue

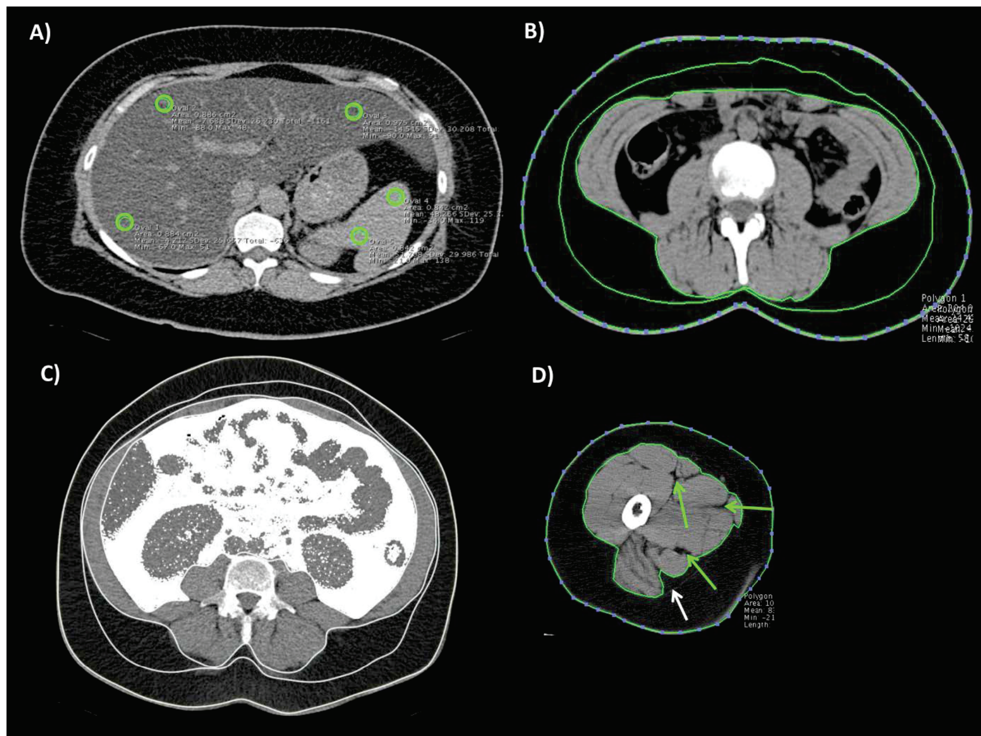


Figure 4: A) CT slice through liver with steatosis and spleen. Regions of interest (ROI) in green circles. B) CT slice through mid-abdomen with green tracings around the superficial and deep subcutaneous adipose tissue compartments. C) CT slice through mid-abdomen. Visceral adipose tissue (-30 to -190 HU) is highlighted in white. D) Adipose tissue in mid-thigh with green tracings around the thigh subcutaneous adipose tissue (TSAT) and the muscle compartment. White arrow points to the fascia, which becomes difficult to visualise further along. Green arrows point to inter-muscular adipose tissue (IMAT). (Wium C. 2013).

Visceral abdominal adipose tissue

The VAT *(Figure 4C)* is situated in the omentum and around the viscera of the intra-abdominal cavity, and is regarded as the most metabolically active abdominal adipose tissue. The amount of VAT has been shown to correlate negatively to insulin sensitivity (75), positively to inflammation (77) and predispose to non-alcoholic fatty liver disease (78) and cardiovascular disease (79).

Adipose tissue in thigh

Adipose tissue in thigh *(Figure 4D)* can be divided into thigh subcutaneous adipose tissue (TSAT), and sub-fascial adipose tissue (SFAT), although the fascia can often be more difficult to visualise than in the abdomen. In addition, there is the inter-muscular adipose tissue (IMAT). Several studies have demonstrated an inverse relation between thigh circumference, TSAT and insulin resistance (80-82), and some, but not all, found a positive relation between SFAT, IMAT and insulin resistance.

Magnetic resonance imaging and magnetic resonance spectroscopy

Magnetic resonance imaging (MRI) is a method for analysing body composition with a high degree of accuracy and without any known long-term side effects. This makes it a method of choice for whole-body analyses, also in children. MRI gives the possibility to analyse regional adipose tissue distribution (83) and also quantify intramuscular adipose tissue (69). Disadvantages include the need for expensive equipment, the relatively long time of analysis in a gantry, which is difficult for people with claustrophobia, and the restricted space. The usual diameter of the MRI gantry is 60 – 70 cm, which excludes the examination of the most obese patients (65). Magnetic resonance spectroscopy (MRS) can be used as a method to discern different chemical entities. Differences in signal intensities can be used to study the distribution of metabolites of interest (65), and MRS has also been used to discern intra-myocellular from extra-myocellular lipid in vivo (70).

1.2 Ethnic differences in type 2 diabetes - Diabetes in South Asians

1.2.1 Ethnicity

Ethnicity and ethnic groups are concepts that are used extensively in medical research. Race was a biological concept that developed in the early 19th century, classifying the human population by physical characteristics. Race nowadays has a negative connotation, and ethnicity is gradually taking over as classification variable. It has been recognised that the genetic and biological variation in racial categorisations is small, making the racial categories misleading in indicating variations in health (84;85). Ethnicity is a complex concept, which has both socio-cultural and biological components. Ethnicity and ethnic groups are used extensively as classifications in studies, but the boundaries between ethnic groups are often unclear, and are usually not well defined in medical literature. There is also an ongoing debate whether the categorisation into ethnic groups is meaningful or misleading in medical research (86;87). Ethnic groups are often categorised through shared origins or social background, culture and distinctive traditions, language and religion. The methods used to categorise study subjects into ethnicities vary, and can for example be the subject's reported self-perception of ethnicity, ethnicity determined from analysis of names, from country of birth, as well as parents' (or even grand-parents') country of birth, from observer determined skin colour, or from other methods (87;88). European studies often use country of birth or parents' country of birth, whereas studies from the UK and the USA tend to use self-perceived ethnicity. In the USA, racial categories, based on the Office of Management and Budget (OMB) classification, are also still used (89).

South Asian ethnicity as a concept is arguably an entity that is much too large and heterogeneous to be useful in medical research, comprising peoples from several countries, with differences in culture, religion and lifestyle. Nevertheless, studies of South Asians in their respective countries of origin, and in immigrant populations to Western countries, have consistently found increased prevalence of diabetes and cardiovascular disease. The use of South Asian ethnicity can therefore be justified, since using this categorisation makes a valuable contribution to bringing medical research forward (86).

1.2.2 Migration

There are two main reasons for migration, the “push factor”, involving negative conditions in the country of origin, such as war and poverty, and the “pull factor”, where prospects of a better life, work opportunities or education draw people to migrate (90). There has been a substantial degree of migration from the South Asian region to Western countries over the last century, due in large part to the region being a former British colony. It has been observed that migration, both from rural to urban areas within South Asia, and to Western countries, with urbanisation and westernisation as a result, has led to lifestyle changes. The changes including an increase in intake of food high in calories and low in dietary fibre (91) and less physical activity (92) has in turn led to an increase in lifestyle related diseases like type 2 diabetes and cardiovascular disease (90). Psycho-social stress related to migration and low socio-economic status might also play a part in development of diabetes and its complications (93), although this is not always shown (94).

Norway experienced an increased rate of immigration from Pakistan in the late 1960s, as other European countries were closing their borders for non-Western immigration. These were labour immigrants, arriving in Norway, mainly in Oslo, in search of work and a better future for themselves and their families in Pakistan. Most of these Pakistani immigrants came from a small, rural area called Kharian in the Gujrat region of Pakistani Punjab (95). The Pakistani immigrants who arrived in the late 1960s and early 70s, until the immigration ban in 1974, were mainly young men who came to work and earn money to send back home, and who planned to return to Pakistan. Many of them nevertheless ended up staying in Norway, and were later joined by wives and other family members. Of the more than 32 000 Pakistani first and second-generation immigrants in Norway today, many still originate from this limited rural area in Gujrat. Eighty-five percent of Pakistani immigrants to Norway live in Oslo and Akershus counties. The Pakistani immigrant group is the largest non-Western immigrant group in Norway, and the largest overall immigrant group in Oslo (96).

The Sri Lankan immigration to Norway has also taken place over several decades, where 1987 was the year with the highest number of Sri Lankans arriving in Norway, 1773 persons (96), probably due to escalations in the civil war between the Sri Lankan (Singhalese) government and the Tamil Tigers. In 2012 there were 14 300 Sri Lankans in Norway, making the Sri Lankans the 16th largest immigrant group (97). The majority of Sri Lankans in Norway are Tamil refugees (96).

1.2.3 Diabetes in South Asian countries

The main countries of the South Asian region, also called the Indian sub-continent, include Pakistan, India, Bangladesh and Sri Lanka. Nepal, Bhutan and the Maldives are also often included in this category. This region has one fifth of all diabetes cases in the world (98), with diabetes occurring in leaner and younger subjects than in Western countries (99). India was until recently the country with the highest total number of subjects with diabetes, now only beaten by China (100). A recent review and meta-analysis of the prevalence and trends of diabetes in South Asia shows a wide variety in the prevalence, from 3.8 % in rural Bangladesh, to 13.9 % in urban India. There is an increasing prevalence trend in all the main South Asian countries, with consistently higher prevalence estimates in urban than rural areas (101). The current national prevalence rates of diabetes are 8.31 % in India, 6.72 % in Pakistan, 9.85 % in Bangladesh and 7.77 % in Sri Lanka (102). According to IDF World Atlas, 14.5 % of all adult deaths in the region are attributable to diabetes, with 55 % of these deaths occurring before the age of 60 and 27 % before the age of 50 years. Diabetes is hence a serious disease also in South Asia, with the second highest mortality rate from diabetes worldwide (103).

As important is the high degree of morbidity due to diabetes complications. The increased prevalence of diabetes in South Asians is an important reason for the high rates of cardiovascular disease (104). Cardiovascular disease (CVD) also occurs at a younger age than in Western populations, with a high mortality rate. CVD deaths in the South Asian population are predicted to increase from 29 % in 2005 to 36 % in 2030 (105). Micro-vascular complications (retinopathy, nephropathy and neuropathy) are linked to poor blood glucose control (106). Data are scarce on the prevalence of these complications in South Asia. Studies of South Asian immigrants to Western countries find higher rates of these complications as well (107;108).

1.2.4 Diabetes in South Asian immigrants to Western countries

In the first half of the 1980s, Mather and Keen and their team performed a house-to-house survey of all residents in the suburb of Southall in West London. There had been some previous reports of high prevalence of diabetes in South Asian immigrants to other parts of the world, like South Africa, Fiji, Trinidad and Singapore, but data on prevalence of type 2

diabetes in South Asian immigrants to Britain were still lacking. Southall was considered a well-integrated, multi-ethnic community, with approximately 66 500 inhabitants, originating from Britain, South Asia, East Africa and the Caribbean. Mather and Keen found an age-adjusted prevalence of diabetes that was at least 3.8 times higher in the group of South Asians and East Africans than in Europeans, and a shift towards lower age in the South Asians with diabetes compared to the Europeans (109).

This marked the start of many more studies of type 2 diabetes in South Asian immigrants to Western countries, both in Europe, America and Australia. The general findings from these studies have been that South Asian immigrants develop diabetes at a younger age (109-111), and at lower BMI(112). They nonetheless have a higher degree of central obesity and lower muscle mass (113;114). They have been found to be more insulin resistant (115;116), have poorer blood glucose control (117;118) and are more prone to diabetes complications, mainly macro-vascular disease, retinopathy, proteinuria and end stage renal disease (119). Numerous publications exist regarding diabetes and insulin resistance in South Asians, both in their countries of origin and in immigrants to Western countries. However, many of the studies have been performed in healthy subjects or subjects with impaired glucose tolerance, and not in subjects with established type 2 diabetes. In addition, many studies use surrogate markers established from fasting measurements of glucose and insulin. Only a few studies have performed euglycaemic clamps to assess insulin resistance, and almost exclusively in healthy subjects (115;120-129). The euglycaemic clamp with isotope tracer measurement of endogenous glucose production in South Asian subjects with type 2 diabetes has, to this author's knowledge, not previously been published.

1.2.5 Diabetes in South Asian immigrants in Norway

Studies from general practice have shown the same tendencies in Norway as in other Western countries, namely higher prevalence of type 2 diabetes in South Asian immigrants, occurring at a younger age, and presenting with impaired blood glucose control compared to native Norwegians (111;130). Diabetes prevalence has been found to be especially high in women of South Asian descent, as high as 26.4 % in Pakistani women and 22.5 % in Sri Lankan women, whereas the prevalence in both Sri Lankan and Pakistani men were approximately 20 %. Prevalence rates in Norwegians were 2.7 % in women and 6.4 % in men (131). Diabetes in this immigrant population is thus becoming an important public health issue also in Norway,

concerning both diabetes care and prevalence of complications. Tran and co-workers have for instance showed a high prevalence of self-reported cardio-vascular disease in ethnic minority subjects with diabetes (15.3%) compared to those without diabetes (5.9%) (132).

1.2.6 Proposed theories for the high diabetes prevalence in South Asians

Thrifty Genotype

As early as in 1962, Neel proposed the thrifty genotype hypothesis (133). This theory focuses on selection of genetic traits favouring energy storage in times of food abundance, for later energy reserves during famine, but which become deleterious in times of permanent excessive energy intake. It is however still unclear whether results from genetic studies support this theory. Recent genetic studies of signals of selection have showed no clear evidence of selection of genes linked to type 2 diabetes or obesity. However, signatures of selection of protective variants have been found (134;135).

Thrifty Phenotype

The finding that low birth weight was strongly associated with increased risk of type 2 diabetes and cardiovascular disease in later life (136), especially when later subjected to over-nutrition (137), lead to the hypothesis of foetal programming and the thrifty phenotype hypothesis (136). This hypothesis emphasises the association between poor foetal and infant growth, inducing epigenetic modifications, with permanent changes in insulin resistance and glucose metabolism, and later development of type 2 diabetes as a result. The hypothesis has been challenged by some inconsistencies. For example: The thrifty phenotype should facilitate energy saving and storage. However, resting metabolic rate has not been found to be consistently lower in people with low birth weight. Regions with cold climate and harsh winters, where there were long periods of food shortage, should have made people more prone to such foetal programming throughout evolution. Yet people from such regions, like the Western Europeans, have been shown to have lower prevalence of insulin resistance (138).

Adipose tissue compartment overflow

The adipose tissue overflow hypothesis (139) proposes the concept that superficial subcutaneous adipose tissue is the primary compartment for fat storage, while deep SAT and VAT are secondary compartments. The latter compartments are less organized and more vascularised than superficial SAT, displaying a higher link to metabolic activity (75;77). In the adipose tissue overflow theory, the superficial SAT is less developed in South Asians compared to white Westerners, rendering them less tolerant to the accumulation of excess energy. With sustained excess energy intake, a subsequent overflow of fat into the deeper depots of more metabolically active deep SAT and VAT occurs (139). These deep fat depots have higher trans-membrane fluxes of NEFA, releasing lipids into both the systemic and portal circulation, and higher secretion of proinflammatory cytokines (5;139). The liver receives an increased influx of NEFA, leading to increases in ectopic fat in the liver and increased hepatic triglyceride and cholesterol synthesis, again leading to atherogenic dyslipidaemia and CVD (139). Increased NEFA in the liver also impairs the inhibition of hepatic glycogenolysis by insulin in the post-prandial state, leading to hyperglycaemia (9;140). Skeletal muscles also receive an increased influx of NEFA from deep SAT and VAT, leading to intra muscular ectopic fat deposition, and increased insulin resistance (9).

Metabolic inflexibility

The metabolic inflexibility theory proposes that the normal switch between high lipid oxidation in the fasting state and high glucose oxidation in the post-prandial or clamp hyperinsulinaemic states is impaired in type 2 diabetes (141). This leads to less lipids being oxidised during fasting, with accumulation of lipids in skeletal muscle as a result, and less glucose being oxidised post-prandially, leading to increased plasma glucose values (141). The degree of metabolic inflexibility is often measured by the ΔRQ between fasting and euglycaemic clamp states. This is however disputed in type 2 diabetes, since the lower ΔRQ is mainly determined by the impaired insulin stimulated glucose disposal rate, and not glucose oxidation (142).

Mitochondrial efficiency

The mitochondrial efficiency hypothesis proposes that South Asians have more tightly coupled mitochondria than Europeans have. This leads to less heat production and higher energy producing efficiency in the mitochondria, thus higher energy conservation and the possibility of weight gain (143). The respiratory chain in mitochondria, where oxidation transforms fuel substrates into energy in the form of ATP, is regulated by energy demand, such that when ADP is not available for phosphorylation, protons cannot enter the mitochondrial matrix through ATP synthase. However, this coupling of substrate oxidation to ATP synthesis is imperfect, with evidence of proton leaks, due to uncoupling proteins (UCP). This proton leak leads to energy dissipation as heat, and thus increased energy expenditure. This could further impact on body weight (144). Uncoupling proteins are members of an anion carrier protein family located in the inner mitochondrial membrane. They reduce metabolic efficiency by promoting net proton translocation from the inter-membrane space into the mitochondrial matrix. The potential energy available for ATP synthesis is thereby reduced (145). UCP1 is mainly expressed in brown adipose tissue. UCP2, however, is expressed in many tissues, while UCP3 is mainly expressed in skeletal muscle. Recent studies have indicated that UCP2 and 3 gene polymorphisms may be associated with obesity, metabolic syndrome and type 2 diabetes in Asian Indians (146-148). A meta-analysis by de Souza et al. published this year also found that the association between some UCP2 and 3 gene polymorphisms and type 2 diabetes was only significant in Asians, and not in people of European ancestry (145). Over-expression of UCP2 has moreover been shown to inhibit glucose-stimulated insulin secretion in rat β-cells and in INS-1 β-cells, through reduced ATP levels, thus providing another link with type 2 diabetes (149).

Others

The variable disease selection hypothesis explains the increased visceral adipose tissue in South Asians by the need for meeting immediate energy demands by the immune system in the gut in situations of gastrointestinal infections (150). Lipid stores that are readily available in the proximity of the gut would therefore be an advantage in areas of endemic gastrointestinal diseases like cholera.

Watve and Yajnik (138) a few years back proposed the behavioural switch hypothesis. This hypothesis consists of a socio-ecological adaptation in South Asians, which induced two

transitions: 1) the transition from “r” to “K”, where “r” is the strategy of producing a large number of offspring, investing little in each, and “K” is a strategy where fewer offspring is produced, but more is invested in each. For example, insulin resistance in pregnant mothers increases the foetal weight, which constitutes an increase of the “investment” in the offspring. 2) The soldier to diplomat transition, where a shift from muscle dependent to brain dependent strategies is advantageous with the advent of more structured societies. Insulin resistance also favours glucose availability for the brain of the diplomat, rather than the muscles of the soldier.

2 Aims

2.1 General aim

The general aim of this study was to explore differences in the pathophysiology of type 2 diabetes in South Asian immigrants to Norway, as compared to Norwegian or Nordic subjects with type 2 diabetes. Better knowledge of the pathophysiology of type 2 diabetes in South Asians in Norway is necessary to develop prevention and treatment strategies that are more efficient.

2.2 Specific aims

To study possible differences between Nordic and South Asian subjects with type 2 diabetes in:

1. insulin sensitivity, through low and high euglycaemic clamp insulin infusions, and by estimation of endogenous glucose production through the tracer dilution method (papers I and III).

2. anthropometrics and body composition (papers I and II).

3. adipokines and inflammation markers and their relation to insulin sensitivity and body composition (papers I and II).

4. β-cell function, through measurement of first-phase insulin secretion during an intra-venous glucose tolerance test (paper III).

5. glucose and lipid metabolism, both in the basal post-absorptive state and during clamp hyperinsulinaemia (paper III).

3 Subjects and Methods

3.1 Patients and design

This thesis is built on results from two clinical studies performed at the Diabetes Research Laboratory, Aker/Oslo University Hospital during the years 2003-2012. The first study, the Diabetes In Pakistani Immigrants (DIPI) project, was a cross-sectional study of Norwegian and Pakistani subjects with type 2 diabetes, where results are presented in papers I and II. The second study, the Diabetes Intervention trial with Vitamin D in subjects of sub-Indian and Nordic Ethnicity (DIVINE) study, was a randomized, controlled intervention trial, which recruited Nordic and South Asian subjects with type 2 diabetes and hypovitaminosis D. Results from the baseline examinations of these subjects are reported in paper III of this thesis.

3.1.1 The Diabetes In Pakistani Immigrants (DIPI) project

Design

This was a cross-sectional study comparing young subjects with type 2 diabetes from two different ethnic groups, Pakistanis immigrants and Norwegians, all living in the Oslo area.

Subjects

We recruited Norwegian and Pakistani patients with type 2 diabetes, aged 45 years or younger. Inclusion criteria were: confirmed type 2 diabetes, subjects of Norwegian or Pakistani origin, age 18 – 45 years, on any type of anti-hyperglycaemic treatment. Exclusion criteria were: ethnicities other than Norwegian or Pakistani, positive test for anti-GAD or anti-IA2 autoantibodies, age > 45 years, person unwilling or unable to give informed consent. We included 19 Pakistani and 21 Norwegian sex-matched patients (age 29-45 years, 49 % men). All Pakistani participants were first generation immigrants. One Pakistani woman was excluded on the first day of testing, because of difficulties in obtaining intravenous access. The remaining 39 patients were examined, where median (IQR) age for the Pakistani and Norwegian subjects were 41 (8) and 42 (6) years respectively, and diabetes duration was 9 (7) and 5 (9) years respectively.

3.1.2 The Diabetes Intervention trial with Vitamin D in subjects of sub-Indian and Nordic Ethnicity (DIVINE) project

Design

The data used in this paper are also cross-sectional, from the baseline data of a randomized controlled intervention trial, designed to investigate the effect of high dose vitamin D supplementation to type 2 diabetic subjects with concomitant vitamin D deficiency or insufficiency. The power estimations were done for the purpose of the intervention trial.

Subjects

Sixty-two patients with type 2 diabetes and vitamin D deficiency or insufficiency, of Nordic or South Asian ethnicity, were recruited from our outpatient clinic, from general practice, from advertising by posters in the hospital lobby and at pharmacies in the region, and from advertisements in local newspapers. Men and women from the Oslo area, above 18 years of age, of Nordic or South Asian origin, were eligible, regardless of type of anti-diabetic treatment. 190 patients were screened, 62 patients were recruited, and 61 patients were subjected to initial intra-venous glucose tolerance tests (IVGTT) and clamp procedures. One of the 62 patients had to be excluded due to severe difficulties in getting the two intravenous catheters in place. The cohort thus consisted of 42 Nordic (65 % men) and 19 South Asian (47 % men) subjects with a mean age of 58.3 ± 8.3 and 49.7 ± 9.4 years respectively, and with a mean diabetes duration of 9.6 ± 7.0 and 9.3 ± 5.4 years.

3.2 Methods

3.2.1 Pre-examination preparations

To create standardised conditions for the clamp examinations, patients in both studies were asked to stop oral anti-diabetic drugs for two days, and insulin for at least 12 hours prior to examination (long-acting insulin analogues for at least 24 hours). Patients were also asked to refrain from strenuous physical exercise and alcohol intake during these two days, and to arrive fasting, including no tobacco, for at least 10 hours, from the night before the

examination. All clamp and IVGTT examinations were performed at the Diabetes Research Laboratory, Oslo University Hospital.

CT measurements in the DIPI-study were performed on a separate day, at the Department of Radiology, Aker University Hospital. The patients had that day eaten a light breakfast between 7.30 and 8.00, and the CT scan was performed at approximately 10.00.

DXA and anthropometrical measurements in the DIVINE study were also performed on a separate day, with DXA measurements at the Bone Laboratory at the Endocrine Outpatient Clinic, Aker, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital. The patients had been asked not to eat or drink for at least 2 hours before arriving for the examinations.

3.2.2 Anthropometrical measurements

Height to the nearest 0.1 cm and weight to the nearest 0.1 kg were measured with participants wearing light clothing and no shoes. Waist and hip circumferences were assessed with a tape measure with spring scale to ensure equal traction at every measurement, measuring at mid-point between the lowest rib margin and the iliac crest, and at the level of the major trochanter, respectively, with subjects standing upright, legs together. BMI was calculated as $\frac{Weight\ (kg)}{Height^2(m)}$. WHR was calculated as waist circumference/hip circumference.

3.2.3 Bioelectrical Impedance Analysis

BIA was performed on a Tanita Body Composition Analyser BC-418 MA (Tokyo, Japan), providing measurements of percentage total body fat, body fat mass in kilograms and fat free mass in kilograms. All subjects were fasting and voided urine before measurement. The standard software algorithm provided by the manufacturer was used for all patients, regardless of ethnicity.

3.2.4 Dual X-ray Absorptiometry

A Lunar Prodigy from GE Healthcare was used for measurement of body composition by dual x-ray absorptiometry, where total fat mass in kg, percentage total body fat and fat free body

mass in kg were measured. Here also, the standard software algorithm provided by the manufacturer was used for all patients, regardless of ethnicity.

3.2.5 CT measurements of adipose tissue compartments

CT measurements of adipose tissue and muscle compartments in the abdomen and thigh were performed using Somatom (Erlangen, Germany) with the patient examined in a supine position, arms extended above the head. Three single axial scans were performed through:

i) the liver and the spleen, at the level of Th12.

ii) the mid-abdomen, 10 cm above L4/L5 in men and 5 cm above L4/L5 in women.

iii) the thighs, at mid-distance between the anterior-superior iliac spine and the upper margin of the patella.

The fat content of the liver was based on attenuation values in Hounsfield Units (HU). The liver-spleen ratio (LS-ratio) was calculated based on the mean of measurements within three regions of interest (ROI), in the liver (two in the right lobe and one in the left lobe), and two ROI measurements in the spleen, each ROI measuring 80 mm^2 *(Figure 4A)*. In mid-abdomen, the circumferences were tracked for the superficial and deep subcutaneous adipose tissue compartments, divided by the superficial fascial plane *(Figure 4B)* (74). The visceral adipose tissue compartment was measured by tracking the inner abdominal circumference, and highlighting the pixels below -30 and above -190 HU in order to quantify only the adipose tissue *(Figure 4C)*.

The right thigh was selected for measuring thigh muscle and superficial adipose tissue compartments. A mark was made on the subject’s thigh at mid-distance between the anterior-superior iliac process and the cranial edge of the patella before examination, to determine the level of the thigh slice. Because of difficulties in tracking the fascia in the thigh, only subcutaneous fat area and muscle area was measured *(Figure 4D)*.

3.2.6 Laboratory measurements

All fasting and non-fasting blood samples from patients were collected from a catheter placed in an elbow vein after an overnight fast.

Fasting plasma glucose was measured immediately at the Diabetes Research Laboratory by the glucose oxidase method. For Paper I and II a Glucose Analyser II (Beckman Instruments) was used, and for Paper III a YSI 2300 (Yellow Springs, OH, USA) was used. On the YSI 2300 whole blood glucose was measured and plasma glucose was calculated with the following equation: whole blood glucose x 1.119, modified from (151). This equation is valid when erythrocyte volume fraction (EVF) is within normal limits.

Serum insulin was collected both fasting and throughout the examinations. After at least 20 minutes coagulation time, the samples were centrifuged and serum separated from blood cells. The samples were then kept refrigerated until analysis. For Papers I and II the radioimmunoassay (RIA) kit, formerly from Linco Research, presently available from Millipore Corp. (Billerica, MA, USA) was used, and for Paper III, both serum insulin and C-peptide were measured using an immuno-fluorometric assay (DELFIA) from Perkin Elmer Life Sciences (Wallac Oy, Turku, Finland). Serum was also collected in the same way, and stored at - 80°C for later 25-hydroxyvitamin D measurements on radioimmunoassay (RIA) kits from DiaSorin (Stillwater, MN, USA). These analyses were all performed at the Hormone Laboratory, Oslo University Hospital.

Lithium-heparin tubes were used for collection of plasma for lipid and transaminase analysis. After immediate centrifugation, the tubes were sent to the Department of Clinical Chemistry for same day analysis. Fasting plasma total cholesterol, HDL cholesterol, triglycerides, ASAT and ALAT were measured using a routine enzymatic method (Roche Diagnostics, Mannheim, Germany). Plasma LDL cholesterol was calculated using the Friedewald equation (152).

EDTA whole blood was collected for same day HbA_{1c} analysis at the Department of Clinical Chemistry, by high performance liquid chromatography. For results reported in Papers I and II the analyses were performed on a Variant analyser (Bio-Rad, Richmond, CA, USA), and for Paper III on a Tosoh G7 analyser (Tosoh Corp., Tokyo, Japan).

EDTA whole blood was also collected for EVF automatic estimation on a Sysmex XE-2100/-5000 (Sysmex Corp., Kobe, Japan), at the Department of Clinical Chemistry.

EDTA-plasma was collected, centrifuged and immediately frozen at – 20°C, and then later in the day transferred to a - 80°C freezer. Plasma for NEFA measurements were collected on chilled EDTA-tubes. NEFA were analysed using a NEFA C enzymatic colour test kit, (Wako Chemicals GmbH, Neuss, Germany), modified to run on a Technicon RA1000 (Technicon Instruments Corp., Tarrytown, NY, USA), at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo.

Plasma levels of adiponectin and leptin were analysed at the Hormone Laboratory using RIA kits from Millipore Corp. (Billerica, MA, USA),(also formerly from Linco Research).

At the Research Institute of Internal Medicine, the plasma high sensitive C-reactive protein (hsCRP) was measured using a DuoSet ELISA kit from R&D Systems (Minneapolis, MN, USA). Plasma interleukin-1 receptor antagonist (IL-1RA) was measured using CytoSet from Invitrogen Corporation (Carlsbad, CA, USA), with streptavidin-horseradish peroxidase from R&D Systems. Plasma measurement of interleukin-6 (IL-6) was performed using a High Sensitivity ELISA kit from Abcam plc. (Cambridge, UK).

Plasma for [6,6-$^{2}H_2$]-glucose analysis was collected on Fluoride Oxalate tubes, immediately centrifuged, separated from blood cells and frozen at – 80°C for later analysis. The [6,6-$^{2}H_2$]-glucose was measured by LC-MS/MS, via turbulent flow chromatography (Cohesive technologies RXT1, Franklin, MA, USA), combined with a tandem mass spectrometry (Sciex API3000, Applied Biosystems, Foster City, CA, USA), at the Clinical Metabolomics Core Facility, (Rigshospitalet, Copenhagen, Denmark).

Urine produced throughout basal equilibration, IVGTT and clamp examinations was collected until the end of the clamp and analysed for urea using an enzymatic-kinetic UV assay on a Roche Modular P analyser (Roche Diagnostic, Basel, Switzerland) at the Department of Clinical Chemistry, Aker, Oslo University Hospital.

3.2.7 Two-step euglycaemic clamp

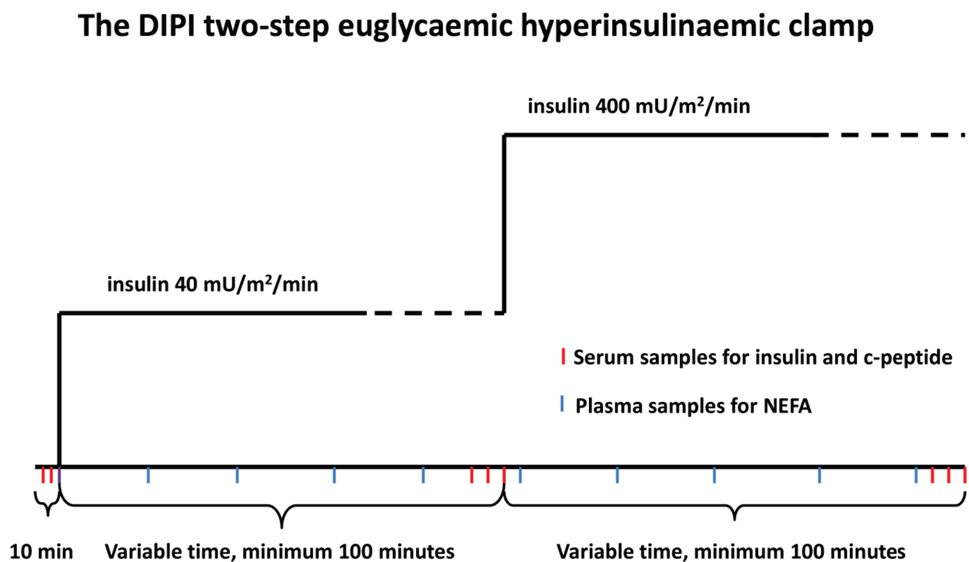
For the DIPI-study, we performed a euglycaemic, hyperinsulinaemic clamp with two steps, first administering an insulin dose of 40 mU/m^2/min for a minimum of 100 minutes, until at least 30 minutes of stable euglycaemia was obtained. This was directly followed by a 400 mU/m^2/min insulin infusion, also for a minimum of 100 minutes, with at least 30 minutes of stable euglycaemia at the end. This gave us euglycaemic clamp measurements with serum

insulin levels both at physiological and at supra-physiological levels. The high-level insulin infusion was included to assure total suppression of endogenous glucose production during clamp.

The glucose infusion rate in µmol/m^2/min was established, and denoted GIR_{40} and GIR_{400} respectively. Because of varying metabolic clearance rates of insulin, the insulin levels, given as the mean of three measurements at ten-minute intervals at the end of each step of the clamp, differed between patients. The insulin sensitivity index was therefore also calculated, and expressed as the ratio of the GIR to the prevailing mean serum insulin levels ((GIR/I) x 100), denoted ISI_{40} and ISI_{400}.

Every 30 minutes during the two-step euglycaemic clamp, EDTA plasma for non-esterified fatty acid (NEFA) measurements was extracted *(Figure 5)*.

Figure 5: The DIPI clamp study



3.2.8 Single step euglycaemic clamp with stable isotope tracer

For the DIVINE-study, we used a single insulin infusion rate, at high-physiological levels, but we also measured the endogenous glucose production, both in the basal, fasting state and during clamp stable euglycaemia. Estimation of endogenous glucose production was performed using the stable isotope dilution method. The stable isotope [6,6-$^2H_2$] glucose was

chosen in order to avoid tracer contamination, and because it was non-radioactive, and thus easier to handle and would not present an obstacle in patient recruitment. The [6,6-$^2H_2$] glucose also is only moderately recycled, in the Cori (lactic acid) and glucose-alanine cycles between muscle and liver, mostly in the basal, post-absorptive state. Estimations of basal endogenous glucose production is therefore reliable, but includes some gluconeogenesis from the Cori cycle, especially in obese type 2 diabetic patients, where this recycling increases total glucose disposal (TGD) approximately 25 % above normal values (52). In the DIVINE-study, all patients had type 2 diabetes and most were obese.

Pre-examination, baseline plasma samples were collected for correction for background [6,6-$^2H_2$] glucose content. A primed (170 mg) continuous (1.7 mg x min^{-1}) infusion of [6,6-$^2H_2$] glucose (Cambridge Isotope Laboratories, Inc., Andover , MA) was initiated in the morning of the examination day, and maintained throughout the experiment. At the end of a 2-hour stabilisation period, collections of three plasma samples at 10-minute intervals were made for measurement of basal endogenous glucose production. At the end of the 2 ½ hour long euglycaemic clamp, another three plasma samples were collected at 10-minute intervals for measurement of clamp endogenous glucose production *(Figure 6)*.

Calculations of endogenous glucose production (EGP) at the end of the basal equilibration period and during clamp euglycaemia were performed. Both were performed during steady state for plasma glucose, with only small variations in glucose concentration and tracer enrichment over time. Thus steady state equations, where rate of appearance equals rate of disappearance, have been applied for the calculation of both EGP and total glucose disposal (153;154). EGP in the basal state was calculated as follows:

$$EGP_{basal} = (\frac{E_i}{E_{p(basal)}} - 1)I$$

where I is the rate of [6,6-$^2H_2$]-glucose infusion (µmol/m^2/min), E_i is the enrichment of the tracer infusion in moles percent excess (mpe) and $E_{p(basal)}$ is the mean [6,6-$^2H_2$]-glucose enrichment in plasma (mpe) at the end of the basal stabilisation period. At the end of the euglycaemic clamp, TGD was calculated as follows:

$$TGD = \frac{I \times E_i + GIR \times E_m}{E_{p(clamp)}} - I$$

where GIR is the exogenous glucose infusion rate (μmol/m^2/min), E_m is the [6,6-$^2H_2$]-glucose enrichment (mpe) in the infused glucose, and $E_{p(clamp)}$ is the mean [6,6-$^2H_2$]-glucose enrichment (mpe) in the plasma samples taken during the last 30 minutes of the clamp euglycaemia. The EGP during clamp euglycaemia, EGP_{clamp} =TGD – GIR. Between subject and within subject coefficients of variation for plasma glucose levels in clamp steady state were 10.0 % and 4.6 % respectively.

3.2.9 IVGTT

After a 2-hour tracer equilibration period, the IVGTT was performed, with a < 1-minute intravenous bolus injection of glucose 500 mg/ml, 0.3 g/kg body weight. Blood samples were drawn for plasma glucose concentration as well as serum insulin and C-peptide determination at -2, 0, 2, 4, 6, 8, 10, 15 and 30 minutes after glucose bolus injection.

The first phase insulin secretion/Acute Insulin Response to glucose (AIRg) was calculated as the incremental area under the curve (AUC) for insulin from time 0-8 minutes and 0-30 minutes.

3.2.10 Euglycaemic clamp with tracer

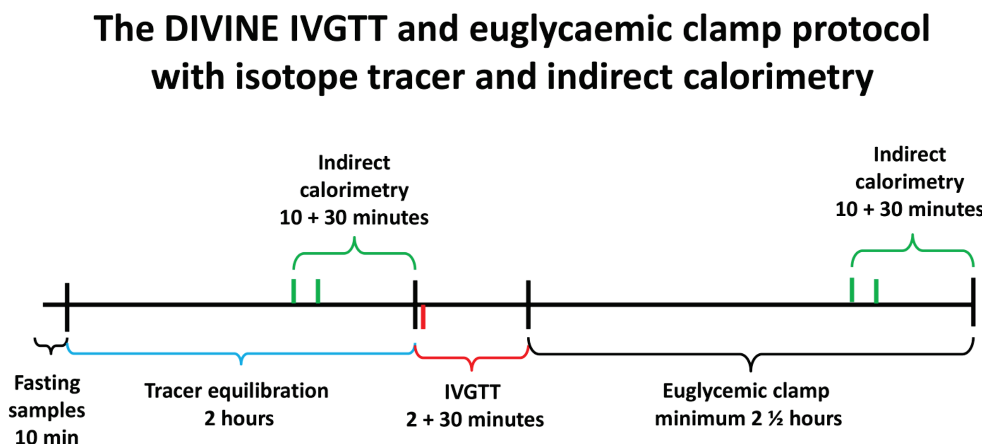
Immediately following the IVGTT, a euglycaemic, hyperinsulinaemic clamp was performed as described in paper III. Insulin was infused at a rate of 80 mU/m^2/min, after an initial bolus and 10 minute priming infusion determined by the patients pre-clamp plasma glucose. The infusion was maintained for 2 ½ hours or more, until at least 30 minutes of stable euglycaemia was obtained. When plasma glucose had decreased to euglycaemic values, a variable infusion of glucose 200 mg/ml enriched with 8 mg x g^{-1} glucose of [6,6-$^2H_2$]-glucose was continually adjusted to maintain euglycaemia.

Plasma glucose was regularly measured on a Precision Xceed glucometer (Abbott Laboratories. Abbott Park, IL), with five-minute intervals when the patient approached euglycaemia. Control measurements at least every 30 minutes, and every 10 minutes during the last 20 minutes of the clamp, were performed on a Y.S.I 2300 STAT analyser. The glucose infusion rate (GIR) in μmol/kg FFM/min was established.

3.2.11 Indirect Calorimetry

Indirect calorimetry measures O_2 uptake from inspired air and CO_2 excretion to expired air, and together with urinary nitrogen losses (measured as urinary urea) enables us to estimate the rate of oxidation of carbohydrates, lipids, and proteins, and measure basal and clamp-hyperinsulinaemic energy expenditure. Together with clamp measurements of total glucose disposal, this gives us information about glucose oxidation and non-oxidative glucose metabolism, as well as lipid oxidation, in both the fasting and clamp hyperinsulinaemic state. Indirect calorimetry was performed using a Jaeger Oxycon Pro (Erich Jaeger, Viasys Healthcare, Germany), a computerized flow-through canopy gas analyser system. During a 40-minute period at the end of the basal tracer equilibration period and at the end of the euglycaemic clamp, the canopy hood was placed over the patient's head. After a ten-minute adaptation period, the expired and inspired air was continuously sampled and analysed for O_2 and CO_2 content during a 30-minute steady state period. Whole body substrate oxidation was estimated from the mean values of VO_2 and VCO_2 measured, and from measurement of urinary nitrogen (urea). Average basal and insulin stimulated glucose and lipid oxidation rates were calculated using Frayn's equations (155). Non-oxidative glucose metabolism was calculated as the difference between total body glucose utilization (as determined by the euglycaemic clamp with tracer dilution method) and the rate of glucose oxidation (as determined by indirect calorimetry).

Figure 6: The DIVINE IVGTT and euglycaemic clamp



3.2.12 Statistical analyses

Data are presented as mean ± standard deviation or median (inter-quartile range) unless otherwise specified. We analysed non-normally distributed data log-transformed, or using non-parametric methods, as appropriate. Student's *t* tests or Mann Whitney U tests were used for comparison of continuous variables between groups, and paired samples *t*-tests were used for within groups analysis of change. For comparison of categorical data between patient groups, the Chi square test for independence was used. Spearman's correlation coefficients (r_s) were used. One–way between-groups ANCOVA was performed, with preliminary checks to ensure no violation of the assumptions of normality, linearity, homogeneity of variances and homogeneity of regression slopes. Multiple linear regression analyses were performed, with log-transformation of parameters when needed, to ensure no violation of the assumptions of normality, linearity and homoscedasticity. In regression analyses Nordic = 1 and South Asians = 2. A two-sided p-value < 0.05 was deemed significant and uncorrected values are presented. In Paper III, Bonferroni-Holm corrections were also performed, and showed that uncorrected p-values < 0.01 remained < 0.05 after correction. Due to the exploratory nature of the studies, with somewhat limited sample sizes, uncorrected p-values were still kept in order to limit type II statistical errors (156). Statistical analyses were performed with SPSS 18.0 and 19.0 for windows (SPSS Inc., Chicago, IL, USA).

3.2.13 Ethical aspects

All participants gave informed consent prior to any study related procedure. The studies were approved by the Eastern/South-Eastern Norway Regional Committee for Medical and Health Research Ethics, and conformed to the declaration of Helsinki of 1964 as revised in 2002 (157) and GCP guidelines (158). The DIVINE study was registered with the US National Library of Medicine Clinical Trials registry, number NCT00992797.

4 Main results – summary of papers

4.1 Paper I

The aim of this paper was to explore possible differences in the pathophysiology of type 2 diabetes in a group of younger Pakistani and Norwegian subjects with type 2 diabetes. The subjects were examined with a two-step euglycaemic, hyperinsulinaemic clamp, with measurements of NEFA every 30 minutes. Fasting blood samples and anthropometrical measurements were performed in all subjects, as well as body composition, assessed by BIA in a subgroup of 30 subjects. The Pakistani subjects had significantly lower BMI, but higher HbA_{1c} and longer diabetes duration than the Norwegians did. We found no statistically significant crude differences in GIR or ISI during the first clamp step, although point estimates were consistently higher in the Norwegian group than the Pakistani group: median (inter-quartile range) GIR_{40}: 468.4 (587.3) vs. 339.8 (468.0) µmol/m2/min ($p = 0.46$), ISI_{40}: 79.7 (137.9) vs. 57.1 (74.1) µmol/m2/min/s-ins ($p = 0.29$). During the second clamp step GIR was still not significantly different, GIR_{400}: 2055.6 (907.0) vs. 1661.1 (672.3) µmol/m2/min ($p=0.08$), whereas ISI_{400} was significantly higher in Norwegians: 20.7 (17.2) vs. 14.2 (7.3) µmol/m2/min/s-ins ($p=0.016$). After adjusting for sex and percentage body fat, a borderline significant lower insulin sensitivity in the Pakistani group was found, both measured as GIR_{40} ($p=0.06$), ISI_{40} ($p=0.012$), GIR_{400} ($p=0.042$) and ISI_{400} ($p=0.014$). We also found that the Norwegian subjects had higher percentage NEFA suppression after 30 minutes of clamp hyperinsulinaemia than Pakistani subjects: 71.2 (42.1)% vs. 41.9 (90.6)%, ($p=0.042$). BMI strongly correlated to insulin sensitivity in the Norwegian group, whereas the Pakistani group was quite insulin resistant regardless of BMI. Waist circumference correlated more strongly to insulin sensitivity in the Pakistani group than the Norwegian group, where the correlation was not significant. Leptin and IL-1RA were negatively associated with insulin sensitivity in the Norwegians but not the Pakistanis. Leptin and IL-1RA correlated negatively to BMI in both Norwegians and Pakistanis and with waist circumference in the Pakistanis only. These findings suggest that there may be important differences in the relationship between obesity, insulin sensitivity and the effect and/or regulation of these signal substances between the two groups. Leptin is known to correlate mainly to the amount of subcutaneous abdominal adipose tissue, and these findings could indicate ethnic differences in the metabolic activity of the different compartments of abdominal adipose tissue.

4.2 Paper II

This paper further examined the relationship between insulin sensitivity, inflammation markers and abdominal fat distribution in the Norwegian and Pakistani groups described in paper I. Abdominal and thigh adipose tissue, liver fat and muscle compartments were examined with computed tomography scans. The study revealed similar areas of adipose tissue in the abdomen between the two groups, but differences in the relationship between adipose tissue compartments and insulin sensitivity as measured by the euglycaemic clamp. VAT correlated significantly negatively to insulin sensitivity in the Pakistani subjects only ($r_s = -0.68$, $p = 0.006$), Norwegians: ($r_s = -0.25$, $p = 0.304$). Plasma levels of leptin were highly correlated to SAT in the abdominal and thigh area in both Norwegians and Pakistanis, with the strongest correlation in the Pakistani group. CRP was positively correlated to abdominal SAT in Pakistani patients only. IL-1RA on the other hand, correlated positively to abdominal and thigh SAT in Norwegian patients only. To conclude, in spite of similar adipose tissue distribution in the Norwegian and Pakistani patients, we found evidence of ethnic differences in the importance of adipose tissue distribution for insulin sensitivity and inflammation, where VAT seemed to be especially important in the metabolic dysregulation in Pakistani patients.

4.3 Paper III

This paper describes more in depth analyses of glucose and fat metabolism in Nordic and South Asian subjects. Insulin sensitivity, insulin secretion and fat distribution as measured by euglycaemic clamp with isotope tracer dilution, indirect calorimetry, IVGTT and DXA were assessed. A group of 62 Nordic and South Asian subjects with type 2 diabetes included in a vitamin D intervention trial were examined at baseline. All participants had hypovitaminosis D. The South Asian group was significantly younger, shorter and leaner than the Nordic group, but had similar diabetes duration and higher HbA1c. The main finding was a significantly higher endogenous glucose production during the post-absorptive state in the South Asian group compared to the Nordic group: 19.1 (9.1) vs. 14.4 (6.8) µmol/kgFFM/min, $p = 0.003$. There were no differences between South Asians and Nordics in total glucose disposal (39.1 ± 20.4 vs. 39.2 ± 17.6, $p = 0.99$) or first phase insulin secretion ($AUC_{0\text{-}8\ min}$: 220 (302) vs. 124 (275), $p = 0.35$). Endogenous glucose production remained detectable in all subjects during clamp hyperinsulinaemia, and could not be reliably predicted by any parameters measured. There was no significant relationship between serum 25-hydroxyvitamin D levels and insulin sensitivity or insulin secretion. There were near significant correlations between resting and clamp energy expenditure and endogenous glucose production in the South Asian group only, which could indicate increased post-absorptive glucose metabolism at the expense of lipid metabolism in the South Asian group, but these results have to be confirmed in larger studies.

5 Discussion

5.1 Methodological considerations

5.1.1 Patient characteristics

Differences in body composition

The patients from the two ethnic groups that have been studied for this thesis display ethnic differences in the degree of adiposity and body composition. We experienced both in the DIPI-study and in the DIVINE- study that the Pakistani/South Asian groups were overall leaner and shorter than the Norwegian/Nordic groups, although we had different inclusion criteria and recruitment strategies in the two studies. This is at odds with the most common findings in literature, when comparing South Asian to Western subjects. The South Asian group tends to be more obese, at least concerning central obesity (113;159). There may be several explanations for this discrepancy.

There is obviously a selection bias in the patients we recruited, since a large part has been recruited from the hospital outpatient clinics. In Norway type 2 diabetes is usually treated in general practice and only the more difficult cases are referred to specialist clinics. These patients may well be more obese than the regular person with type 2 diabetes, at least in the Norwegian/Nordic population. This may not necessarily be the case for the South Asians, though. We have seen that this ethnic group also in general practice often presents with poorly regulated diabetes (111). The South Asian patients referred to specialist clinics are therefore not necessarily very different from those treated in general practice.

Another explanation for the discrepancies with other studies is the choice of inclusion criteria. We have chosen to examine established type 2 diabetic patients with median disease duration of 5 to 9 years in the various groups, whereas most of the studies published on insulin resistance in South Asians have in fact examined healthy or pre-diabetic subjects (113;128;160-162).

We have shown that the South Asian group is shorter and leaner, but find no difference in total percentage body fat, or in the areas of abdominal adipose tissue or ectopic fat infiltration.

The areas of muscle tissue in abdomen and thigh did tend to be lower in Pakistani patients, consistent with findings from other authors (114). The similar size of abdominal adipose tissue compartments in the two ethnicities might also be due to a selection of more obese Norwegian patients.

Differences in HbA_{1c}

HbA_{1c} has been consistently higher in the South Asian compared to the Nordic groups, both in the DIPI-study where the groups were similar in age, but where the Pakistani patients had longer disease duration, and in the DIVINE-study where the South Asians were significantly younger than the Nordic patients, but where disease duration was similar. This indicates that the South Asian patients have a disease that is more difficult to control, either owing to differences in quality of diabetes care, perhaps because of language barriers or cultural differences, or due to inherent differences in disease pathophysiology. Tran et al. have presented a study of quality of care for patients with diabetes in general practice in Oslo. In this study, different ethnic groups were found to have received comparable quality of diabetes care, but ethnic minority groups like the South Asians had earlier onset of diabetes and poor glycaemic control. This indicated a need for tighter follow-up and collaboration between general practice and specialist health care (111). This is in line with our findings, pointing towards higher insulin resistance, especially hepatic insulin resistance, with increased fasting endogenous glucose production, leading to more hyperglycaemia and higher HbA_{1c}.

Differences in age

The patient groups studied in the DIPI-study and DIVINE-study differed in age through differences in inclusion criteria. This might be a reason for the different results seen in the measurements of insulin sensitivity.

In the DIPI-study, the participants were selected according to age between 18 and 45 years. The two ethnic groups were similar in age, between 29 and 45 years old. Even in this young group of diabetes patients, the Pakistani group had significantly longer diabetes duration than the Norwegian group. In the DIVINE-study, there was no upper age limit at inclusion. The Nordic group ended up older than the South Asian group by approximately 10 years. However, the two groups in the DIVINE-study had similar diabetes duration.

5.1.2 Measurements of body composition

We have performed dual x-ray absorptiometry measurements and/or bioelectrical impedance analyses of body composition in both studies. It was known that DXA prediction models for fat mass and lean body mass established in a healthy white population could be less appropriate in other populations with a different body composition (163) however, this had not been properly investigated in South Asian men and women at the time we examined them. Taylor et al. have recently published a study validating DXA measurements of abdominal fat in Indians against MRI measurements (164). They found good overall agreement between DXA and MRI measurements, but there was evidence of an overestimation of fat mass by DXA at low values of abdominal fat, and in females. This strengthens the validity of our DXA measurements, with the reservation that Taylor et al. used a different DXA instrument than ours. For BIA there was growing evidence that ethnic specific algorithms should be used in BIA measurements. A few algorithms were published on South Asians/Asian Indians at the time of examination (68;165;166), however none using the Tanita analyser, and they proved difficult to use on our BIA registrations, with uncertainties regarding comparability with the BIA registrations in the Nordic participants. We therefore decided to use the standard algorithms embedded in the analyser software in measurements of all our participants. Waidyatilaka and co-workers has recently published a study validating a BIA equation in Sri Lankan urban women (66).

The discrepancies that might exist in the measurement of body composition by DXA, especially in the leanest women, might have moderately influenced the results in the South Asian group, where fat free mass might have been somewhat underestimated. Both fasting and clamp endogenous glucose production as well as total glucose disposal is expressed per kg fat free mass, measured by DXA. A systematic error in the estimation of fat free mass in lean South Asian women concerns only a few of the 61 subjects, and would therefore hardly have any influence on the data on insulin resistance and glucose metabolism.

5.1.3 Hepatic glucose production

Many clamp studies are performed without measuring endogenous glucose production, although there is evidence that endogenous glucose production is not totally suppressed, at least in type 2 diabetic, insulin resistant subjects (56). In the DIPI-study, we did effectively experience difficulties in lowering blood glucose levels to euglycaemia during the first step of

the clamp in a number of patients, signifying that endogenous glucose production was not suppressed. We therefore used an approach with two steps of hyperinsulinaemia, to be able to measure insulin sensitivity in everybody. This method does however have some limitations. We found that it became impossible to complete the highest step of the clamp in some cases, where the subjects had near-normal insulin sensitivity, because the amount of glucose needed for the infusion to balance the hyperinsulinaemia exceeded recommended doses, and risked creating phlebitis. In addition, the highest step of the clamp induced serum insulin levels of 10,000 pmol/l or more, which is very far from physiological insulin levels. Our experiences from the DIPI-study therefore lead us to conclude that we needed to measure endogenous glucose production in the DIVINE-study. The results from the DIVINE-study in fact showed a non-negligible endogenous glucose production during clamp, which was impossible to estimate through surrogate markers. This has further confirmed the need for measuring endogenous glucose production during euglycaemic clamps in subjects with insulin resistance, so as not to underestimate the total glucose disposal.

5.1.4 Choice of equation for calculation of total glucose disposal

The most widely used method for calculating total glucose disposal by isotope tracer dilution is Steele's equation for non-steady state glycaemia (167), here modified to account for addition of tracer to the variable glucose infusion (168):

$$R_a(t) = \left[\frac{E_b}{E_p(t)} - 1\right] I - \frac{pVG(t)\left(\frac{dE_p(t)}{dt}\right)}{E_p(t)} + \frac{E_{var} \times I_{var}}{E_p(t)}$$

We chose to opt for a simpler equation, which produced results that were comparable to results using Steele's equation, but which did not produce negative values of total glucose disposal rates. These negative values are an artefact of Steele's equation, which in fact is an equation for a single pool model used in a multiple pool system. It is a well-known phenomenon, and, of course, not physiologically possible. Our experience had been that even regular analytical variability in the deuterated glucose measurements during the periods of steady state euglycaemia created variations in $dE_p(t)/dt$ of Steele's equation that became so important that this in several cases lead to negative values of EGP, although the actual variation in tracer concentration was negligible. This was seen both at the end of the basal and the clamp examinations. In addition, the use of a pool fraction, and the size of this pool

fraction is debated (169). As it turns out, when the $dE_p(t)/dt$ is low, then the pool fraction has little overall effect. We had minimized the variation in tracer to tracee ratio by adding tracer to the variable glucose infusion during the euglycaemic clamp (168). All measurements of total glucose disposal and endogenous glucose production were made in conditions of steady state, where the variation in plasma glucose was minimal. We therefore deemed it appropriate to use an equation for steady state:

$$TGD = \frac{I \times E_i + GIR \times E_m}{E_{p(clamp)}} - I$$

where I is the rate of $[6,6\text{-}^2H_2]$-glucose infusion (µmol/m^2/min), E_i is the enrichment of the tracer infusion in moles percent excess (mpe), GIR is the exogenous glucose infusion rate (µmol/m^2·min), E_m is the $[6,6\text{-}^2H_2]$-glucose enrichment (mpe) in the infused glucose, and $E_{p(clamp)}$ is the mean $[6,6\text{-}^2H_2]$-glucose enrichment (mpe) in the plasma samples taken during the last 30 minutes of the clamp euglycaemia.

5.1.5 Normalising the glucose infusion rates/total glucose disposal rates

The recommended way to obtain standardised indices of insulin sensitivity is to normalise the M-value (glucose infusion rate) for fat free mass, especially in obese subjects or in groups where both sexes are represented, to account for differences in body fat mass between subjects (170). In the DIPI-study, we unfortunately lacked data on fat free mass in approximately ¼ of the patients. The glucose infusion rate was therefore normalised for body surface area. We also showed results both non-normalised and normalised for serum insulin values at each euglycaemic clamp step, to account for differences in insulin clearance and the ability of exogenous insulin to suppress endogenous insulin secretion. According to De Fronzo et al (171), this insulin sensitivity index, a measure of the amount of glucose metabolised per unit of insulin concentration, should be an appropriate way to express insulin sensitivity. However, the importance of normalising for serum insulin levels is disputed, since it has been demonstrated that the high variability in mean GIR values observed in various studies of normal-weight, non-elderly, non-diabetic subjects becomes even greater when normalised to serum insulin (172). Also, the clearance rate of insulin becomes non-linear above a certain level of serum insulin (approximately 3500 pmol/l), and in these supra-physiological circumstances it is difficult to know the level of insulin action on peripheral

tissues (172). In the DIVINE-study, we therefore chose to normalise the total glucose disposal for fat free mass only, and not for serum insulin levels.

5.1.6 Differences in obesity and the relation to adipokines and inflammation

A limitation to our studies was that the Norwegian/Nordic groups were significantly more obese than the Pakistanis/South Asians. In the DIPI-study, this could have attenuated possible differences in adipokines and markers of inflammation, such that they became impossible to discern, given the limited group sizes as well. There was no difference in leptin levels. However, with the higher total body fat mass in Norwegians, one would have expected higher leptin levels in this group, since leptin is mostly produced in subcutaneous adipose tissue (22), the largest of the adipose tissue compartments. This could signify that matching the groups for degree of obesity could have shown higher leptin levels in the Pakistani group. Adjusting for parameters of obesity did, however, not unmask such a difference in our material. The point estimates of sTNF-R1 and adiponectin, are lower in the Pakistani group than in the Norwegian group, although the difference does not reach statistical significance (p=0.14 and 0.15 respectively). Adiponectin shows no association with adipose tissue compartments or total body fat. There was only an indirect association with insulin sensitivity, (data not shown in paper I); when dividing patients into two groups according to adiponectin levels higher or lower than median, we found significantly higher ISI_{400} levels in the high adiponectin group compared to the low adiponectin group in the Norwegian patients, (24.1 (11.2) vs. 13.2 (12.6), *p=0.048*), but not in the Pakistani patients, (15.6 (19.8) vs. 12.8 (6.1), *p=0.841*).

5.1.7 Sample size and power

In the DIPI-study, we calculated sample size and power from available results in earlier studies, which were performed in other diabetes patient populations, mainly older. These results, in fact, underestimated the variance in the clamp measurements of insulin sensitivity. The variance, especially in our Norwegian group of young type 2 diabetic individuals, was much higher than anticipated. The DIPI-study therefore turned out somewhat underpowered.

The DIVINE-study was an intervention trial, where power calculations were performed for the main outcome, which was change in insulin sensitivity from baseline to end of trial in the treatment group compared to the placebo group. Recruitment was focused on the intervention

study, and no matching of the two ethnic groups concerning age, HbA_{1c} or body composition was made. We had intended to include 30 patients from each ethnic group, but the recruitment of South Asian patients proved more difficult than anticipated, leading us to reduce the South Asian group and increase the Nordic group. It was also more difficult to get the South Asian subjects to undergo a complete set of examinations. Some patients declined the indirect calorimetry measurements due to claustrophobia from the canopy hood. The South Asian group was therefore more limited than first intended was.

5.2 Main findings

5.2.1 Insulin sensitivity

The results from the DIPI study, which showed reduced insulin sensitivity in the Pakistani group, are in line with findings in other studies in South Asian subjects with type 2 diabetes (125;173). The DIPI study was performed without measurement of endogenous glucose production, and therefore did not measure true total glucose disposal, only the exogenous glucose infusion rate. In the DIVINE-study, we performed more thorough examinations of total glucose disposal and glucose metabolism, including measurements of endogenous glucose production, something that has not been published previously in South Asians with type 2 diabetes. We found significantly higher post-absorptive endogenous glucose production in the South Asian group. Endogenous glucose production during clamp hyperinsulinaemia was, however, not significantly different in the two groups, although endogenous glucose production given as a percentage of total glucose disposal tended to be higher in South Asians. After adjustment for central adiposity, in the form of waist-to-height ratio, this percentage became significantly higher in the South Asians, signifying higher hepatic insulin resistance.

Contrary to our expectations, we found no ethnic difference in total glucose disposal in the DIVINE-study. The South Asian group, in particular, is of limited size in the DIVINE study. It is possible that we could have found a difference in total glucose disposal given a larger study sample, and if the two ethnic groups had been matched for body composition. Adjusting for waist circumference, amongst other factors, did increase the difference in TGD between the groups, although no significant difference was unveiled.

It is also possible that the main ethnic difference in insulin resistance in fact lies primarily in the higher hepatic insulin resistance in South Asians. Hepatic insulin resistance impairs insulin's ability to suppress endogenous glucose production. The need for exogenous glucose infusion during clamping would naturally be much lower in South Asians, where a relatively higher rate of endogenous glucose production persisted. In view of the fact that the peripheral glucose uptake did remain similar in the two ethnic groups, this implies that the level of peripheral insulin sensitivity is the same in the two ethnic groups. If this is the case, a larger focus on the hepatic glucose and lipid metabolism in the South Asian population is warranted, to elucidate possible causes, and thereby develop better treatment strategies.

Increased hepatic insulin resistance can be caused by excess NEFA, which inhibit the insulin receptor signalling pathways, and prevent the insulin mediated inhibition of gluconeogenesis and glycogenolysis (5). We have seen in the DIPI study that NEFA suppression during euglycaemic clamp hyperinsulinaemia was significantly impaired in the Pakistani group.

The adipose tissue overflow hypothesis constitutes one intriguing explanation for excess NEFA in the liver (139). The "overflow" of lipids to the more metabolically active compartments, i.e. deep subcutaneous and visceral adipose tissue increases their metabolic activity even more, thus releasing more NEFA into the circulation, with transportation to the liver. In the DIPI study we have seen that DSAT and VAT correlated significantly to insulin sensitivity in Pakistani, but not in Norwegian subjects, in accordance with this hypothesis. The NEFA flux to the liver and peripheral tissues also increases ectopic fat depositions. We have not seen increased fatty infiltration in the liver in South Asian groups. However, in the DIPI-study we also showed a significant correlation between the insulin sensitivity index and liver attenuation in the total patient group. The correlation coefficient in the Pakistani group was of similar magnitude as in the total group, but did not reach significance, probably due to the smaller group size.

With possibly increased levels of NEFA in the liver, and perhaps also in skeletal muscle, the metabolic inflexibility hypothesis can come into light (141). As previously stated, NEFA suppression during hyperinsulinaemic clamp was significantly impaired in Pakistani subjects in the DIPI-study. A sign of metabolic inflexibility is blunting of the usually rapid decline in plasma NEFA following the first phase insulin secretion at the beginning of a meal (174). If we transfer this to the euglycaemic clamp setting, this is in line with our findings in the DIPI-study. We do have data from meal tests in this cohort, which will be interesting to look into

further. With regard to the muscular fuel switch, we have not performed any measurements of pure skeletal muscle metabolism, such as limb balance methods (141), so the evidence of metabolic inflexibility in muscle will have to be circumstantial.

5.2.2 Glucose metabolism

In the DIVINE-study, we were able to study glucose metabolism in more detail, both looking at endogenous glucose production and further metabolism through oxidative and non-oxidative glucose disposal. We were limited by the low number of South Asians who underwent indirect calorimetry measurements. However, we did find indications, although not statistically significant, that the higher endogenous glucose production in the South Asian group resulted in higher both oxidative and non-oxidative glucose metabolism in the post-absorptive state. In the post-absorptive state, the organism mainly uses lipids as energy substrate. However, with increased plasma glucose levels, glucose will compete with NEFA as substrate for energy production, hence the oxidative glucose metabolism will increase. There is also some excess glucose and NEFA which has been metabolised in muscle into trioses/lactate/pyruvate. Instead of entering the citric acid cycle, they are returned to the liver through the Cori cycle or the glucose-alanine cycle to become substrates for gluconeogenesis. This implies that the post-absorptive hyperglycaemia participates in its own self-maintenance by the non-oxidative recycling, and is reinforced by hyperlipidaemia, where non-oxidative degradation of NEFA also becomes a substrate for gluconeogenesis in the liver. Our findings are thus in line with the theory of adipose tissue overflow, and of metabolic inflexibility, with increased use of glucose as energy substrate in the fasting condition, due to hyperglycaemia.

Is there evidence in our studies for increased mitochondrial efficiency in the South Asian subjects? We have in fact measured lower resting energy expenditure in South Asians, although this was mainly due to lower FFM. We have so far not analysed any polymorphisms of the UCP 2 or 3 genes. Our groups are probably too limited in size to find ethnic differences.

5.2.3 Body composition

One of the hypotheses for the difference in prevalence of type 2 diabetes between the Western European and South Asian population in Western countries has been a difference in body composition, leading to a higher degree of insulin resistance. Several studies have shown that

South Asians were more abdominally obese, with higher waist circumferences or larger abdominal adipose tissue compartments, correlating to measures of insulin sensitivity (113;114;159;175;176). However, most of these studies have been performed in non-diabetic subjects, and used surrogate markers for insulin resistance, like the HOMA IR. In our two studies, most of our subjects were obese, both in the South Asian and in the Nordic groups, although the Nordic subjects were significantly more obese than the South Asians, both regarding waist circumference and BMI. Interestingly, when comparing abdominal adipose tissue compartments in the two ethnic groups in the DIPI-study, there were no significant differences, neither in area of subcutaneous nor visceral adipose tissue. The degree of fatty infiltration in the liver was also similar. Still, the Pakistani subjects in the DIPI-study had lower insulin sensitivity than the Norwegian subjects, and VAT was strongly negatively correlated to insulin sensitivity in the Pakistani group only. One way to interpret this finding is that it is not the amount of adipose tissue per se that is important for insulin resistance and diabetes risk. There must be one or even several other factors making the central obesity in South Asians more deleterious than in Western Europeans. The fact that visceral adipose tissue was correlated to insulin sensitivity in the Pakistani and not in the Norwegian group supports this. We therefore investigated the role of several adipokines and inflammation markers, to see if they could represent this factor, discussed in a later section.

Another possible explanation for the higher insulin sensitivity in the Norwegian group, despite higher levels of obesity and similar areas of abdominal adipose tissue compartments, is that in the DIPI-study, we recruited young subjects who had been diagnosed with diabetes in early adulthood, which is more uncommon in the Norwegian than in the Pakistani population. It is possible that these selected young Norwegians have a different kind of diabetes, where obesity and other traditional risk factors are less important, and where a genetic or other inherited susceptibility is the most important factor. We did however also observe a highly significant correlation between insulin sensitivity and BMI in the Norwegian group only. From GWAS studies it is recognized that few susceptibility genes for type 2 diabetes that until now has been identified, are involved in obesity and insulin resistance (*FTO*, *PPARG*), whereas the large majority involve insulin secretion and β-cell function (48). In the Norwegian group, the range of insulin sensitivity was much larger, with several subjects having near normal values, but still they had developed type 2 diabetes. We cannot exclude that some of these subjects do not have typical type 2 diabetes, and may have

monogenic diabetes. They were, however, older at time of diagnosis than the typical patient with monogenic diabetes was. (177).

5.2.4 Inflammation

We wanted to investigate whether the difference in diabetes prevalence could be caused by a higher degree of low-grade inflammation in the South Asians, contributing to higher insulin resistance. Several other studies have reported lower levels of adiponectin, higher levels of leptin, CRP and other inflammation markers in South Asians with or without diabetes (113;178-180). In the DIPI-study, several adipokines/inflammation markers were measured, but we found no ethnic differences in plasma levels of these substances. There were however, differences in the relationship between these adipokines and insulin resistance and body composition, where leptin and IL-1RA correlated negatively with insulin sensitivity in the Norwegian group only. These adipokines are mainly linked to subcutaneous adipose tissue, indicating that SAT might have a more important role in insulin sensitivity in the Norwegian group than in the Pakistani group, perhaps due to the higher degree of obesity. Adiponectin also seemed to play a more important role for insulin sensitivity in Norwegians than in Pakistanis, contrary to previous reports. Leptin correlated significantly positively with SAT in both abdomen and thigh in Norwegian and Pakistani patients, while IL-1RA only correlated with SAT in Norwegians. CRP on the other hand, significantly correlated to SAT only in the Pakistani group. Surprisingly, none of the adipokines/inflammation markers correlated with VAT, and there was no significant correlation between adipose tissue compartments and IL-6. This seems to indicate that the importance of VAT for insulin resistance in Pakistanis is not mediated by the markers we have measured. There might of course be other inflammation markers involved, that we have not measured. One could also speculate that NEFA could be an important factor, although fasting NEFA values did not differ between the two groups. These were however analysed in peripheral plasma, and not in the portal circulation.

5.2.5 β-cell function

In the DIVINE-study, we assessed the first phase insulin secretion through intravenous glucose tolerance tests. We did not find any significant ethnic difference in the acute insulin response to glucose (AIRg). However, the groups were of limited size, and first phase insulin secretion only gives us part of the picture regarding β-cell function. Contrary to the

euglycaemic hyperinsulinaemic clamp for insulin resistance, there is no gold-standard method to investigate insulin secretion and β-cell function (181). The subjects all had established type 2 diabetes, where AIRg is often said to be lost (182). Still, we found some increase in insulin AUC in 53 out of 60 subjects, and two thirds had AIRg above 100 pmol x min/l.

As previously mentioned, insulin resistance could also affect the β-cell, leading to β-cell dysfunction through inhibition of glucose mediated insulin secretion (6). In the DIPI-study, the Pakistani subjects had a non-significant tendency towards lower fasting serum C-peptide than the Norwegians, and in the DIVINE-study, the South Asians had significantly lower fasting C-peptide than the Nordic subjects, despite a tendency towards higher fasting plasma glucose. This lower fasting insulin production could also be a contributing factor for the higher basal, post-absorptive endogenous glucose production. The raised fasting endogenous glucose production, and increased hyperglycaemia could in part be explained by β-cell dysfunction resulting in inadequate insulin secretion. However, adjusting the EGP for fasting C-peptide did not eliminate the ethnic difference in EGP.

More pronounced impaired β-cell function has been reported in South Asians with gestational diabetes (GDM). Kousta et al. showed lower HOMA-B and lower AIRg than controls in South Asian post-GDM women (183). Mørkrid et al. showed that South Asian women with GDM increased in insulin resistance from gestational week 15 to week 28, similar to Norwegian women, but their β-cell function (HOMA-B) increased significantly less, and hence did not match the change in insulin resistance (184).

6 Conclusions and clinical implications

6.1 Conclusions

The main conclusions of this thesis are:

- South Asian subjects with type 2 diabetes have a higher degree of hepatic insulin resistance.
- Whether an ethnic difference in peripheral insulin resistance also exists, has not been completely elucidated by these studies.
- The Nordic subjects in both studies were more obese than the South Asians. Despite this, there were no significant differences in the size of adipose tissue compartments. The muscle compartments in abdomen and thigh were however smaller in the Pakistani women, and abdominal muscle compartment also bordered on smaller in Pakistani men.
- The visceral adipose tissue, although not larger, was more metabolically active, with a strong correlation to insulin resistance in South Asians.
- There were no differences in blood levels of adipokines/markers of inflammation measured. The relationship between adipokines and adipose tissue compartments could not explain the ethnic difference in insulin resistance.
- There was no ethnic difference in β-cell function measured by first phase insulin secretion during IVGTT. However, fasting C-peptide was lower in South Asians despite higher fasting plasma glucose, indicating a possibility of impaired basal insulin secretion.
- South Asians had significantly higher basal endogenous glucose production.
- South Asians had lower resting energy expenditure than Nordics, mainly due to lower amount of fat-free body mass.

6.2 Clinical implications

We have shown that South Asian subjects have higher endogenous glucose production, signifying hepatic insulin resistance. Whether peripheral insulin resistance also is higher is still uncertain. This should be kept in mind when treating this patient group. The general advice of lifestyle modifications and metformin as the basis of treatment in type 2 diabetes should indeed also apply to these patients, and they should possibly be kept on metformin treatment for as long as possible, to counteract some of the hepatic insulin resistance.

We have moreover shown that visceral adipose tissue is correlated to insulin resistance in the South Asian group. It would therefore be natural to target weight loss as an important means of improving insulin sensitivity, although we have not shown directly in our studies that weight loss improves insulin sensitivity in this group.

Both the DIPI and the DIVINE-study showed higher HbA1c in the Pakistani/South Asian groups; in line with Tran's findings in her study of quality of diabetes care in general practice (111). This underlines the importance of treating this group of patients aggressively, to achieve acceptable goals of glucose control.

6.3 Further research

There is a need for further studies of insulin, glucose and lipid metabolism in South Asians, to verify the results of this thesis, and further explore the aetiology of insulin resistance in this ethnic group.

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8 Papers and manuscripts

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BMC Endocrine Disorders

RESEARCH ARTICLE Open Access

Differences in insulin sensitivity, lipid metabolism and inflammation between young adult Pakistani and Norwegian patients with type 2 diabetes: a cross sectional study

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Abstract

Background: Immigrants from South Asia to Western countries have a high prevalence of type 2 diabetes mellitus (T2DM). We explored pathogenic factors that might contribute to the high risk of T2DM in Pakistani immigrants to Norway.

Methods: A cross-sectional study was performed in 18 Pakistani and 21 Norwegian men and women with T2DM (age 29 – 45 years), recruited from two hospital out-patient clinics. Anthropometrics and a two-step euglycemic, hyperinsulinemic clamp with measurements of non-esterified fatty acids (NEFA) during clamp, was performed in all patients. Insulin sensitivity, given as the Glucose Infusion Rate (GIR) and Insulin Sensitivity Index (ISI), was calculated from the two euglycemic clamp steps. Fasting adipokines and inflammatory mediators were measured. Continuous variables between groups were compared using Student's *t* test or Mann–Whitney *U* test as appropriate. Spearman's correlation coefficient and multiple linear regression analyses were used.

Results: Despite having a lower BMI, Pakistani patients were more insulin resistant than Norwegian patients, during both low and high insulin infusion rates, after adjustment for sex and % body fat: median (interquartile range) $GIR_{(low\ insulin)}$: 339.8(468.0) vs 468.4(587.3) μmol/m^2/min ($p = 0.060$), $ISI_{(low\ insulin)}$: 57.1(74.1) vs 79.7(137.9) μmol/m^2/min ($p = 0.012$), $GIR_{(high\ insulin)}$: 1661.1(672.3) vs 2055.6(907.0) μmol/m^2/min ($p = 0.042$), $ISI_{(high\ insulin)}$: 14.2(7.3) vs 20.7(17.2) μmol/m^2/min ($p = 0.014$). Pakistani patients had lower percentage NEFA suppression 30 minutes into clamp hyperinsulinemia than Norwegians: 41.9(90.6)% vs 71.2(42.1)%, ($p = 0.042$). The relationship of ISI to BMI, leptin and interleukin-1 receptor antagonist also differed between Norwegians and Pakistanis.

Conclusions: Compared with Norwegian patients, Pakistani patients with T2DM had lower insulin sensitivity, affecting both glucose and lipid metabolism. The relation of insulin sensitivity to BMI and some adipokines also differed between the groups.

Keywords: Ethnicity, South Asian, Insulin sensitivity, Anthropometry, NEFA, Adipokines, Inflammation

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Background

Immigrants from South Asia to Western countries have a high prevalence of Type 2 diabetes mellitus (T2DM) [1-4]. The prevalence is also high in urban areas in their countries of origin [5-7]. Studies comparing non-diabetic subjects from India and the US indicate that Indians have lower insulin sensitivity [8,9]. Norway, like many other Western countries, has a growing population of immigrants, with one of the largest groups being of Pakistani origin. This group has an alarming prevalence of T2DM, manifesting at a younger age than in ethnic Norwegians [10], and reaching more than 25% in 30–60 year old South Asian women living in an Eastern suburb of Oslo [11]. Better knowledge through studies of the pathophysiology of T2DM in this population is necessary to develop more efficient prevention and treatment strategies. Such studies could also give new insight into the pathogenesis of T2DM in general.

There is a well known association between obesity and T2DM, but South Asians develop T2DM at lower levels of body mass index (BMI) than Westerners, and are more insulin resistant for any given BMI [12,13]. Several studies have indicated that, in individuals from South Asia, BMI may be inferior to other measures of adiposity as a predictor of metabolic risk [13-15].

Adipokines and inflammatory mediators derived from adipose tissue may be important in the pathogenesis of insulin resistance and T2DM [16]. Possible differences in the profile of these substances between South Asians and Westerners with T2DM, could potentially help explain differences in the development of T2DM in these groups.

In the present study, we investigated a group of young adult immigrants from Pakistan with T2DM, and compared them to a group of Norwegian patients of similar age. We chose to study young adult subjects, where disease duration was relatively short, to avoid comorbidities that might confound the interpretation of our data. Our aim was to explore differences between the groups with regards to pathogenic factors such as insulin resistance, obesity and inflammation, which might contribute to the high prevalence of T2DM in Pakistani immigrants to Norway.

Methods

Design

This was a cross-sectional study comparing young T2DM patients from two different ethnic groups.

Patients

Norwegian and Pakistani patients with T2DM, aged 45 years or younger, attending the out-patient clinics at Lovisenberg Deaconess Hospital between 1999 and 2005, and Aker University Hospital between 2003 and 2009,were eligible for inclusion. After searches in the two hospitals' patient registries, 195 patients were randomly selected to receive an invitation to participate in the study. Exclusion criteria were: ethnicities other than Norwegian or Pakistani, positive GAD or IA2 auto-antibodies, age > 45 years, person unwilling or unable to give informed consent. Patients were contacted by letter with information about the study in Norwegian and Urdu, and patients whose telephone number could be obtained were also informed by phone. Nineteen Pakistani and 21 Norwegian sex-matched patients (age 29–45 years, 49% men) with confirmed T2DM, agreed to participate, and were included in the study. Participant characteristics were similar to a subgroup of approximately 80 of the 155 patients not included, where data on HbA_{1c} and anthropometrics were available. All Pakistani participants were first generation immigrants. One Pakistani woman was excluded on the first day of testing, because of difficulties in obtaining intravenous access. The remaining 39 patients were examined.

Anthropometrical measurements

Height and weight were measured with participants wearing light clothing and without any shoes on. Waist and hip circumferences were assessed with a tape measure at mid point between the lowest rib margin and the iliac crest, and at the level of the major trochanter, respectively. Bioelectrical impedance analysis (BIA) was performed on a Tanita Body Composition Analyzer BC-418 (Tokyo, Japan). All subjects were fasting and voided urine before measurement.

Euglycemic clamp

To enhance comparability of examinations, all patients stopped taking oral antidiabetic drugs for two days, and insulin for at least 12 hours prior to examination. Patients were asked to refrain from strenuous physical exercise and alcohol intake during these two days, and to fast from midnight during the night before the examination. We performed a euglycemic, hyperinsulinemic clamp, using a modification of the method originally described by De Fronzo et al. [17]. The clamp was performed with two steps, first administering a primed, continuous insulin dose of 40 $mU/m^2/min$ for a minimum of 100 minutes, until at least 30 minutes of stable euglycemia was obtained. This was directly followed by a 400 $mU/m^2/min$ insulin infusion, also for a minimum of 100 minutes, with at least 30 minutes of stable euglycemia at the end. The body surface area was calculated using Mostellers equation [18].

Human insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) 300 mU/mL and Glucose 200 mg/mL were infused through a teflon catheter in a vein at the left elbow of the patient. Insulin was diluted in NaCl 0.9%, after having first added 2 ml of the patients own blood,

to avoid insulin sticking to the walls of the bag. All blood samples were drawn from a teflon catheter in a vein at the right elbow, kept open by a slow infusion of NaCl 0.9%. The right arm was kept at 37°C by a heating sleeve connected to a thermal control unit (Swetron AB, Veddestad, Sweden), to arterialize blood samples. Plasma glucose was measured every five minutes using a OneTouch Ultra glucose measuring device (LifeScan, Milpitas, CA), with control measurements every 30 minutes by the glucose oxidase method, on a Glucose Analyzer II (Beckman Instruments, Fullerton, CA). At the end of each step of the clamp, three measurements of serum insulin were taken at ten-minute intervals. The glucose infusion rate in μmol/m^2/min was established, and denoted GIR_{40} and GIR_{400} respectively. Because of varying metabolic clearance rates of insulin, the insulin levels measured at the end of each step of the clamp differed between patients. The insulin sensitivity index (ISI) was therefore also calculated, and expressed as the ratio of the GIR to the prevailing mean serum insulin levels ((GIR/I) × 100), denoted ISI_{40} and ISI_{400}. Every 30 minutes during the 2 step euglycemic clamp, EDTA plasma for non-esterified fatty acid (NEFA) measurements was extracted and immediately frozen at −70°C.

Two Norwegian and two Pakistani patients did not attain euglycemia during the first step of the clamp, and were excluded from this part of the clamp analyses. Two Norwegian and four Pakistani patients were not examined with the high-step clamp, due to contraindication of hyperosmolar glucose at high infusion rates (for the most insulin sensitive patients) and long duration of the low-step part of the clamp to reach euglycemia (for the most insulin resistant patients). After excluding these patients from clamp analyses, there were 46% men in the first step and 48% men in the second step of the clamp.

Blood samples

Fasting plasma glucose was measured by the glucose oxidase method on a Glucose Analyzer II (Beckman Instruments). Serum insulin was analyzed using the radioimmunoassay (RIA) kit, formerly from Linco Research Inc. (St. Charles, MO), presently available from Millipore Corp. (Billerica, MA). Plasma HbA_{1c} was measured by high performance liquid chromatography on a Variant analyzer (Bio-Rad, Richmond, CA). Fasting serum total cholesterol, serum HDL cholesterol, and serum triglycerides were measured using a routine enzymatic method (Roche Diagnostics, Mannheim, Germany). Serum LDL cholesterol was calculated using the Friedewald equation [19]. NEFA were analyzed using a NEFA C enzymatic color test kit, (Wako Chaemicals GmbH, Neuss, Germany), modified to run on a Technicon RA1000 (Technicon Instruments Corp., Tarrytown, NY). Plasma levels of adiponectin and leptin were analyzed using RIA kits from Millipore Corp. (Billerica, MA),(also formerly from Linco Research Inc. (St. Charles, MO)). Plasma measurements of soluble tumor necrosis factor-receptor type 1 (sTNF-R1) and high sensitive C-reactive protein (hsCRP) were performed using DuoSet ELISA kits from R&D Systems (Minneapolis, MN). Plasma measurements of interleukin-1 receptor antagonist (IL-1RA) were performed using CytoSet from Invitrogen Corporation (Carlsbad, CA), with streptavidin-horseradish peroxydase from R&D Systems. Plasma measurements of interleukin-6 (IL-6) were performed using a High Sensitivity ELISA kit from Abcam plc. (Cambridge, UK).

Statistical analysis

Data are presented as mean ± SD, or median (interquartile range) as appropriate. We analysed non-normally distributed data log-transformed, or with the use of nonparametric methods. Student's *t* test or Mann Whitney U tests were used for comparison of continuous variables between groups. For correlations, Spearman's correlation coefficient (r_s) was used. Multiple linear regression analyses were performed with log-transformation of parameters when needed, to obtain normally distributed residuals. A two-sided p-value <0.05 was considered significant, but owing to the large number of comparisons, particular attention should be directed towards analyses where p-values are <0.01. Statistical analyses were performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, IL).

Ethics

The study was carried out in accordance with the Helsinki Declaration, and approved by the Eastern Norway Regional Committee for Medical and Health Research Ethics. Informed, written consent was obtained from each participant before enrolment.

Results

Ethnic differences in diabetes duration and anthropometrics

The two ethnic groups did not differ significantly in age, sex and fasting plasma glucose. In contrast, BMI and weight, including both total body fat mass and lean body mass were significantly higher in the Norwegian, compared with the Pakistani group (Table 1). Despite lower BMI, Pakistani patients had higher median HbA_{1c}, longer median duration of diabetes, and a tendency towards earlier onset of diabetes (reported mean age at onset 30 years vs 34 years, $p = 0.06$). Further clinical characteristics of the participating patients are presented in Additional file 1.

Ethnic differences in insulin sensitivity

When examining insulin sensitivity with a two-step euglycemic, hyperinsulinemic clamp, median values for GIR and ISI showed consistently higher point estimates in the Norwegian compared to the Pakistani group,

Table 1 Clinical characteristics of patients according to ethnic group

	Norwegians	Pakistanis	
	n = 21	n = 18	p
Males (%)	10 (48%)	9 (50%)	
Age (years)	42 (6)	41 (8)	0.865
Years with diabetes	5 (9)	9 (7)	**0.021**
Weight (kg)	106.8 ± 13.6	90.1 ±23.4	**0.009**
BMI (kg/m^2)	37.2 (6.0)	30.9 (9.4)	**0.008**
Waist circumference (cm)	114.3 ± 10.9	106.5 ±17.4	0.102
Waist-hip ratio	1.00 ± 0.09	1.01 ± 0.09	0.575
Total body fat (%)	36.9 ± 9.6	34.2 ± 7.7	0.395
Total body fat mass (kg)	39.5 ± 12.0	28.0 ± 8.2	**0.005**
Lean body mass (kg)	67.1 ± 12.6	54.2 ± 12.2	**0.007**
Fasting plasma glucose (mmol/l)	10.7 ± 3.2	10.6 ± 3.3	0.973
HbA_{1c} (%/mmol/mol)	7.3/56 (1.4/13)	8.7/72 (2.9/31)	**0.022**
Fasting insulin (pmol/l)	166 (160)	209 (193)	0.405
Fasting C-peptide (pmol/l)	1162 ± 458	977 ± 373	0.193
Total cholesterol (mmol/l)	4.5 ± 1.0	4.7 ± 1.3	0.512
HDL cholesterol (mmol/l)	1.03 ± 0.21	1.08 ± 0.24	0.557
LDL cholesterol (mmol/l)	2.7 ± 0.8	2.6 ± 0.6	0.868
Triglycerides (mmol/l)	1.6 (1.1)	1.4 (1.1)	0.832

Table 1: For normally distributed parameters, mean ± SD is given, and comparisons were made using Student's *t* test. For non-normally distributed parameters, median (interquartile range) is given, and comparisons were made using the Mann–Whitney *U* test. Median HbA_{1c}-values are reported as NGSP-%/IFCC-mmol/mol with interquartile range in parentheses. In bold: $p < 0.05$.

although we found no statistically significant crude differences between the groups in GIR or ISI during the first step (Table 2). However, when adjusting for sex and % TBF in a multiple regression analysis, $LogGIR_{40}$ showed a clear tendency towards lower values in the Pakistani group, and $LogISI_{40}$ was significantly lower in the Pakistani compared to the Norwegian group (Table 3). Further adjustment for BMI, HbA_{1c} or diabetes duration did not change the relation of ISI_{40} or GIR_{40} to ethnicity. Ethnicity, sex and % TBF together explained 55% of the variation in insulin sensitivity, expressed as ISI_{40}. During the second step of the clamp, Pakistani patients had non-significantly lower median GIR_{400}, and significantly lower ISI_{400} (Table 2). In standard multiple regression analyses, ethnicity and % TBF explained 29% of ISI_{400} and 24% of GIR_{400} variation (Table 3). Sex was no longer a significant predictor of $LogISI_{400}$ or Log GIR_{400}, and neither was BMI, diabetes duration or HbA_{1c}.

Ethnic differences in the relation of BMI and waist circumference to insulin sensitivity

When exploring the relationships between anthropometric characteristics and insulin sensitivity, we found a

Table 2 Insulin sensitivity and NEFA suppression

	Norwegians	Pakistanis	p
GIR_{40}	468.4 (587.3)	339.8 (468.0)	0.456
ISI_{40}	79.7 (137.9)	57.1 (74.1)	0.289
S-insulin $_{end\ low\ step}$ (pmol/l)	546 (336)	715 (243)	**0.041**
GIR_{400}	2055.6 (907.0)	1661.1 (672.3)	0.080
ISI_{400}	20.7 (17.2)	14.2 (7.3)	**0.016**
S-insulin $_{end\ high\ step}$ (pmol/l)	9505 (2738)	11082 (2922)	0.069
Fasting p-NEFA (mmol/l)	0.59 (0.43)	0.50 (0.29)	0.394
NEFA suppression 0–30 minutes (%)	71.2 (42.1)	41.9 (90.6)	**0.042**

Table 2: Data are presented as median (interquartile range), and comparisons are made using the Mann–Whitney *U* test. In bold: $p < 0.05$. GIR_{40} = glucose infusion rate$_{low\ step}$, ISI_{40} = glucose infusion rate/s-insulin$_{low\ step}$, GIR_{400} = glucose infusion rate$_{high\ step}$, ISI_{400} = glucose infusion rate/s-insulin$_{high\ step}$. GIR is expressed as μmol/m^2/min, and ISI as 100 × μmol/m^2/min/s-Ins. NEFA = non-esterified fatty acids. Number of Norwegians and Pakistanis were: during the low step, n = 19 and 16, respectively. During the high step, n = 19 and 14, respectively, and for NEFA measurements, n = 13 and 15, respectively.

strong, significant correlation between ISI_{40} and BMI in the Norwegian group, and a weaker, non significant correlation in the Pakistani group. Waist circumference, however was significantly correlated with ISI_{40} in the Pakistani, but not in the Norwegian group (Figure 1a-b and Additional file 2). There was also a significant interaction between ethnicity and LogBMI *($p = 0.032$)* in a multiple regression analysis, suggesting a difference between the ethnic groups. (Dependent variable: $LogISI_{40}$, independent variables: ethnicity *(p = 0,026)*, LogBMI *(p = 0,004)*, as well as ethnicity x LogBMI). We were, however, not able to confirm an ethnic difference in the relationship between insulin sensitivity and waist circumference by regression analysis (data not shown).

Ethnic differences in suppression of non-esterified fatty acids

There were no significant differences in fasting NEFA values between Norwegian and Pakistani patients, but initial NEFA suppression by hyperinsulinemia, expressed as percentage suppression of NEFA after 30 minutes of the clamp, was significantly more pronounced in the Norwegian group compared to the Pakistani group (Figure 2, Table 2).

Adipokines and markers of inflammation

Table 4 shows levels of adipokines and markers of inflammation in the two ethnic groups. The Norwegian, but not the Pakistani group, showed negative correlations between ISI_{40} and leptin, as well as IL-1RA (Additional file 2). Fasting IL-1RA was significantly positively correlated to BMI in both patient groups. In the Norwegian group it was also positively correlated to

Table 3 Multiple regression analyses of insulin sensitivity

Variable	Adjusted effect β	95% CI	p-value	Adjusted effect β	95% CI	p-value
Model A:	**$LogISI_{40}$**			**$LogGIR_{40}$**		
Ethnicity	−0.335	(−0.588, -0.081)	**0.012**	−0.210	(−0.429, 0.010)	0.060
Sex	−0.726	(−1.129, -0.322)	**0.001**	−0.455	(−0.804, -0.106)	**0.013**
% body fat	−0.061	(−0.084, -0.037)	**<0.001**	−0.044	(−0.065, -0.024)	**<0.001**
Model A + BMI	0.008	(−0.015, 0.031)	0.464	0.002	(−0.017, 0.022)	0.803
Model A + HbA_{1c}	−0.009	(−0.095, 0.078)	0.835	−0.038	(−0.111, 0.036)	0.300
Model A + Diabetes duration	−0.001	(−0.028, 0.026)	0.941	0.005	(−0.019, 0.028)	0.697
Model B:	**$LogISI_{400}$**			**Log GIR_{400}**		
Ethnicity	−0.186	(−0.333, -0.040)	**0.014**	−0.117	(−0.229, -0.005)	**0.042**
% body fat	−0.009	(−0.017, 0.000)	**0.040**	−0.007	(−0.013, 0.000)	**0.043**
Model B + Sex	−0.163	(−0.394, 0.068)	0.158	−0.081	(−0.262, 0.101)	0.369
Model B + BMI	0.005	(−0.007, 0.018)	0.378	0.005	(−0.004, 0.014)	0.295
Model B + HbA_{1c}	0.005	(−0.045, 0.055)	0.829	−0.013	(−0.051, 0.025)	0.493
Model B + Diabetes duration	−0.008	(−0.023, 0.007)	0.304	−0.007	(−0.019, 0.005)	0.231

Table 3: Dependent variables in model A: $LogISI_{40}$ or $LogGIR_{40.}$ Constant for Log ISI_{40} = 4.428. R^2: 0.55. Constant for $LogGIR_{40}$ = 4.464. R^2: 0.48. Reference group for ethnicity = Norwegians, for sex = women. Variables excluded from final models: BMI, HbA_{1c}(NGSP) and diabetes duration.
Dependent variables in model B: $LogISI_{400}$ or $LogGIR_{400.}$ Constant for Log ISI_{400} = 1.650. R^2: 0.29. Constant for $LogGIR_{400}$ = 3.558. R^2: 0.24. Variables excluded from final models: sex, BMI, HbA_{1c} and diabetes duration. In bold: $p < 0.05$.

leptin ($r_s = 0.59$, $p = 0.006$). In the Pakistani group, IL-1RA neither correlated to ISI, nor to leptin ($r_s = 0.14$, $p = 0.593$), but correlated positively to waist circumference.

Discussion

In the present study we found significant differences in insulin sensitivity, measured by the euglycemic, hyperinsulinemic clamp, between young adult Norwegian and Pakistani patients with T2DM. There were also ethnic differences in diabetes duration and HbA1c, even in this young patient population, demonstrating longer duration of diabetes and poorer glycemic control in the Pakistani group. These differences, however, did not explain the lower insulin sensitivity in the Pakistani group per se, since neither HbA_{1c} nor diabetes duration were significantly associated with the ethnic differences found in insulin sensitivity in adjusted multiple regression analyses.

Furthermore, we demonstrated a different relationship between insulin sensitivity and BMI in the two groups. Although obesity is clearly an important factor in insulin resistance, BMI was a poor marker of metabolic disturbances in the Pakistani patients. Whereas Pakistani patients were all quite insulin resistant regardless of BMI, among the Norwegian patients there was a strong negative correlation between BMI and insulin sensitivity. To our knowledge, this is the first study, using the euglycemic clamp to measure insulin sensitivity, to show such ethnic differences in subjects with T2DM. Our results support and expand previously published results obtained in healthy South Asian immigrants [20,21].

NEFA are normally suppressed by hyperinsulinemia. There is evidence that this suppression is impaired in T2DM [22], although this has not been found by all authors [23]. Our study shows similar fasting NEFA values between the two ethnic groups, but significantly slower NEFA suppression in the Pakistani group, despite higher fasting and end-clamp serum insulin levels. Some evidence of ethnic differences, both in fasting NEFA levels and degree of suppression by hyperinsulinemia, has previously been shown in non-diabetic subjects [24]. Other authors, however, found no difference in NEFA suppression according to ethnicity [25]. Our findings further support that impaired insulin sensitivity affects lipid metabolism more severely in Pakistani patients as compared to Norwegian patients with T2DM.

It is noteworthy that leptin and IL-1RA were differently related to insulin sensitivity in the two ethnic groups. T2DM is now regarded as a disorder characterized by non-resolving inflammation. IL-1, released though activation of Nod-like receptor protein 3 (NLRP3) inflammasomes, seems to play an important role in the inflammatory processes [26]. Il-1RA is considered an anti-inflammatory cytokine, that antagonizes IL-1β and IL-1α, and is elevated, at least in part, in response to elevation of these inflammatory cytokines. An early study showed decreased IL-1RA levels in type 2 diabetes [27], and it has been demonstrated that Leptin decreases β-cell production of IL-1RA, down-regulating IL-1RA expression in pancreatic β-cells in type 2 diabetes [28]. However, more recent studies have shown high levels of IL-1RA in obesity, correlating with BMI, insulin resistance and serum

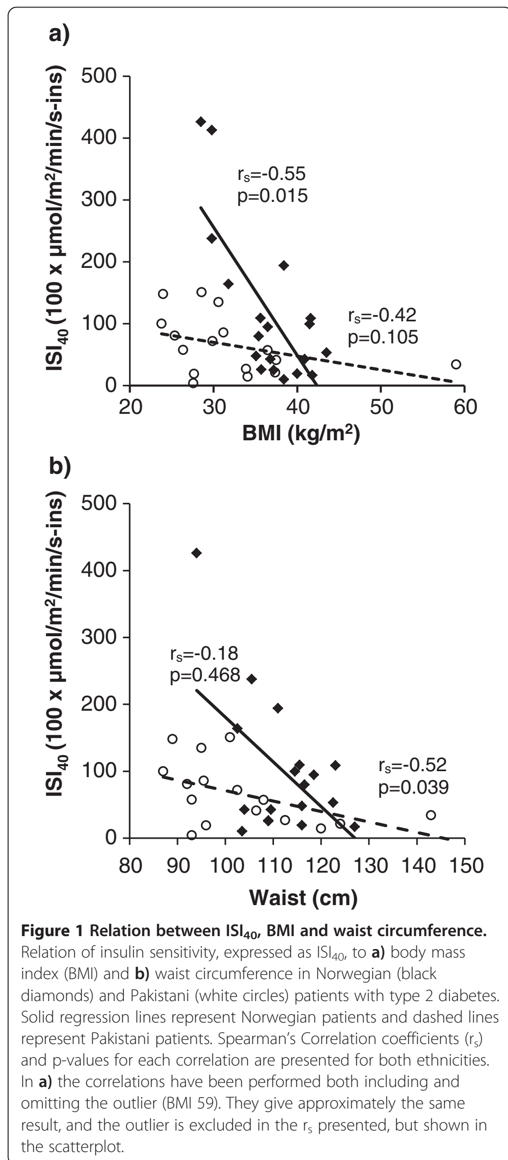

Figure 1 Relation between ISI_{40}, BMI and waist circumference. Relation of insulin sensitivity, expressed as ISI_{40}, to **a)** body mass index (BMI) and **b)** waist circumference in Norwegian (black diamonds) and Pakistani (white circles) patients with type 2 diabetes. Solid regression lines represent Norwegian patients and dashed lines represent Pakistani patients. Spearman's Correlation coefficients (r_s) and p-values for each correlation are presented for both ethnicities. In **a)** the correlations have been performed both including and omitting the outlier (BMI 59). They give approximately the same result, and the outlier is excluded in the r_s presented, but shown in the scatterplot.

leptin levels, and increased production of IL-1RA in adipose tissue in obese humans [29-31]. Subjects with impaired glucose tolerance have higher levels of IL-1RA [32], and in two prospective cohort studies, IL-1RA was found to be elevated several years before diabetes diagnosis, and significantly predicted incident diabetes [33,34]. Whether this represents a counteracting mechanism in response to

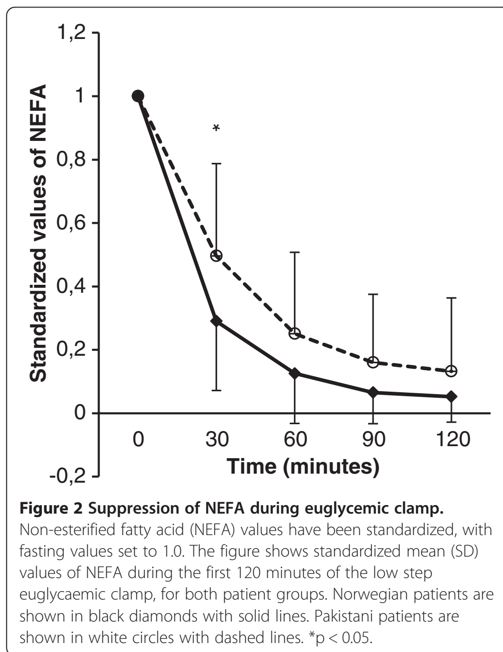

Figure 2 Suppression of NEFA during euglycemic clamp. Non-esterified fatty acid (NEFA) values have been standardized, with fasting values set to 1.0. The figure shows standardized mean (SD) values of NEFA during the first 120 minutes of the low step euglycaemic clamp, for both patient groups. Norwegian patients are shown in black diamonds with solid lines. Pakistani patients are shown in white circles with dashed lines. *$p < 0.05$.

IL-1, which circulates at much lower levels in plasma and is more difficult to detect in clinical samples, is at present unclear. In our study, leptin as well as IL-1RA was negatively associated with insulin sensitivity in the Norwegian, but not the Pakistani patients. Leptin and IL-1RA correlated with BMI in both groups, and with waist circumference in the Pakistani group only. These findings, together with the different relationship between insulin sensitivity and BMI demonstrated in our two groups, suggest that there may be important differences in the relationship

Table 4 Adipokines and markers of inflammation

	Norwegians	Pakistanis	*p*-value
Adiponectin (µg/ml)	4.6 (4.4)	3.3 (3.4)	0.15
Leptin (pmol/l)	950 (1043)	907 (1014)	0.89
IL-6 (pg/ml)	1.18 (1.19)	1.07 (2.18)	0.43
hsCRP (pg/ml)	2.31 (3.48)	2.45 (2.46)	0.63
IL-1RA (pg/ml)	62.75 (98.10)	45.90 (103.95)	0.36
sTNF-R1 (ng/ml)	1.04 (0.20)	0.92 (0.31)	0.14

Table 4: Data are presented as median (interquartile range), and comparisons are made using the Mann–Whitney *U* test. Nor = Norwegian patients. Pak = Pakistani patients.The number of Nor/Pak were: for adiponectin and leptin, n = 21/18. For IL-6, hsCRP and IL-1RA, n = 20/17, and for sTNF-R1, n = 20/16. IL-6 = interleukin-6, hsCRP = high sensitive C-reactive protein, IL-1RA = interleukin-1 receptor antagonist, sTNF-R1 = soluble tumor necrosis factor-receptor 1.

between obesity, insulin sensitivity, and the effect and/or regulation of these signal molecules between the two ethnic groups. As leptin is known to correlate mainly to superficial abdominal adipose tissue [35,36], our findings could indicate that ethnic differences exist in the metabolic activity of the different compartments of abdominal adipose tissue.

The main strengths of our study include thorough patient examinations with gold standard methods for measurement of insulin sensitivity, and using two steps of hyperinsulinemia. The difference in HbA_{1c} and diabetes duration between the two patient groups may be regarded as a weakness. However, these differences were also present in patients who were not included in the study, and have been demonstrated in several other studies [10-12]. Thus, an attempt to match for these parameters could in our opinion have created a selection bias. It can nevertheless be regarded as a limitation to our study that patients were recruited from a population referred to hospital outpatient diabetes clinics, and that sample size was limited, increasing the risk of type II statistical errors. Recruitment of patients, especially the Pakistani patients, proved challenging, which explains why only 20% of the invited patients participated. While a larger and more representative cohort would have strengthened our study, we note that the ethnic differences in insulin sensitivity showed consistent patterns for all four estimates of insulin sensitivity. This also corresponds well with the clinical picture of higher diabetes prevalence and poorer glycemic control apparent in South Asian populations. We did not measure endogenous glucose production during the euglycemic clamp, and our glucose infusion rates may therefore underestimate the true peripheral glucose uptake, at least during the low insulin infusion level. Lastly, body composition was measured by BIA, which may be less accurate than dual x-ray absorptiometry, computed tomography or magnetic resonance imaging. BIA is, however, an inexpensive and accessible way of estimating body composition, which is gaining use in clinical practice.

Conclusions

In this study of Norwegian and Pakistani patients with T2DM we found significant differences in insulin sensitivity and the relationship between insulin sensitivity and obesity markers, which may impact on our understanding of the pathogenic mechanisms that place Pakistani subjects at higher risk of developing T2DM. Further studies are needed to elucidate a possible relationship between insulin sensitivity, various adipose tissue compartments and adipokines and related molecules in various ethnic groups.

Additional files

Additional file 1: Table showing. Further clinical characteristics of patients according to ethnic group.

Additional file 2: Table showing. Correlations between parameters of insulin sensitivity, anthropometry, adipokines and inflammation.

Abbreviations

Anti-GAD: Anti-glutamic acid decarboxylate; Anti-IA2: Anti-protein tyrosine phosphatase; BIA: Bioelectrical impedance analysis; BMI: Body-mass index; ELISA: Enzyme linked immunosorbent assay; GIR_{40}: Glucose infusion rate at the low insulin infusion clamp step; GIR_{400}: Glucose infusion rate at the high insulin infusion clamp step; hsCRP: High sensitivity C-reactive protein; IL-1α: Interleukine-1 alpha; IL-1β: Interleukine-1 beta; IL-1RA: Interleukine-1 receptor antagonist; IL-6: Interleukine-6; ISI_{40}: Insulin sensitivity index at the low insulin infusion clamp step; ISI_{400}: Insulin sensitivity index at the high insulin infusion clamp step; NEFA: Non-esterified fatty acids; NLRP3: Nod-like receptor protein 3; RIA: Radio immuno assay; sTNF-R1: Soluble tumor necrosis factor-receptor 1; SD: Standard deviation; T2DM: Type 2 diabetes mellitus; %TBF: Percent total body fat.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CW conceived of the study, participated in its design and coordination, researched data and wrote the manuscript. ETA researched data and critically revised the manuscript. TU performed analyses of inflammation markers and contributed to discussion. AEM performed analyses of inflammation markers. PMT participated in the design of the study and revised the manuscript. IFL conceived of the study and participated in patient recruitment. PAT provided the insulin and adipokine analyses and revised the manuscript. PA contributed to discussion and critically revised the manuscript. KIB conceived of the study, participated in its design, contributed to discussion and critically revised the manuscript. All authors read and approved the final manuscript.

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Additional file 1: Further clinical characteristics of patients according to ethnic group.

	Norwegians n = 21	**Pakistanis** n = 18
Diabetes treatment n(%)		
Lifestyle ± OAD / Insulin ± OAD	11 (52%) / 10 (48%)	5 (28%) / 13 (72%)
Other medications		
Statins	10 (48%)	4 (22%)
Blood pressure lowering agents	8 (38%)	7 (39%)
Self reported complications		
Macrovascular	2 (10%)	3 (17%)
Retinopathy	2 (10%)	3 (17%)
Nephropathy or microalbuminuria	2 (10%)	6 (33%)
Neuropathy	1 (5%)	3 (17%)
Diabetic foot	1 (5%)	0 (0%)
Others (ED, fatty liver, periodontitis)	5 (24%)	6 (33%)
Co-morbidities		
Astma/COPD	4 (19%)	4 (22%)
Psychiatric conditions	4 (19%)	1 (6%)
GI disease	5 (24%)	2 (11%)
Other endocrine disorders	2 (10%)	3 (17%)
Smoking	10	1
Parity in women		
0-2	11	4
3-5	0	5

Supplementary Table 1: Parity was defined as the number of live-born children each woman had given birth to. OAD = oral antidiabetic drugs. ED = erectile dysfunction. COPD = chronic obstructive pulmonary disease. GI = gastro-intestinal.

Additional file 2: Correlations between parameters of insulin sensitivity, anthropometry, adipokines and inflammation

	ISI_{400}		**BMI**		**Waist**		**% Fat**		**Adiponectin**		**Leptin**		**IL-6**		**hsCRP**		**TNF-R1**		**IL-1RA**	
	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak	Nor	Pak
ISI40 (r_s)	**0.76**	**0.65**	**-0.55**	-0.42	-0.18	**-0.52**	-0.42	**-0.62**	0.37	-0.06	**-0.52**	-0.16	0.05	0.15	-0.22	-0.31	-0.21	0.31	**-0.72**	0.00
p-values	**<0.001**	**0.017**	**0.015**	0.105	0.468	**0.039**	0.102	**0.024**	0,121	0.837	**0.022**	0.549	0.847	0.589	0.361	0.265	0.384	0.288	**0.001**	0.990
ISI 400 (r_s)			-0.27	-0.09	-0.15	-0.21	-0.18	**-0.56**	0.35	0.04	-0.39	-0.06	0.07	0.11	-0.07	-0.22	0.12	0.03	**-0.49**	-0.16
p-values			0.270	0.759	0.570	0.474	0.516	**0.047**	0.139	0.887	0.098	0.840	0.773	0.697	0.791	0.455	0.632	0.929	**0.039**	0.603
BMI (r_s)					**0.54**	**0.92**	**0.62**	0.41	-0.15	0.04	**0.68**	**0.56**	0.02	0.03	-0.17	0.28	0.36	-0.23	**0.50**	**0.49**
p-values					**0.017**	**<0.001**	**0.008**	0.144	0.551	0.874	**0.001**	**0.016**	0.925	0.900	0.487	0.273	0.116	0.386	**0.026**	**0.045**
Waist (r_s)							0.17	0.34	-0.10	-0.08	0.27	**0.48**	-0.12	0.03	-0.14	0.33	0.37	-0.19	0.30	**0.625**
p-values							0.520	0.230	0.681	0.760	0.264	**0.042**	0.637	0.911	0.567	0.202	0.125	0.478	0.206	**0.007**
% Fat (r_s)									-0.21	0.37	**0.82**	**0.86**	-0.06	0.08	-0.28	0.33	0.25	-0.50	0.48	-0.08
p-values									0.411	0.196	**<0.001**	**<0.001**	0.829	0.793	0.294	0.245	0.343	0.085	0.058	0.799

Supplementary table 2: Spearman's correlation coefficients (r_s) with p-values. Correlations with $p<0.05$ are shown in bold. Nor=Norwegian patients. Pak=Pakistani patients. ISI_{40}=glucose infusion rate/s-$insulin_{low\ step}$, ISI_{400}=glucose infusion rate/s-$insulin_{high\ step}$. BMI=body mass index. Waist=waist circumference. % Fat=percentage total body fat. IL-6=interleukin-6, hsCRP=high sensitive C-reactive protein, sTNF-R1=soluble tumor necrosis factor-receptor 1, IL-1RA=interleukin-1 receptor antagonist.

II

Adipose tissue distribution in relation to insulin sensitivity and inflammation in Pakistani and Norwegian subjects with type 2 diabetes

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Running Head: Diabetes, ethnicity and adipose tissue

Abstract

Immigrants from South Asia to Western countries have a high prevalence of type 2 diabetes mellitus (T2DM) associated with obesity. We investigated the relationship between diabetes and adipose tissue distribution in a group of younger T2DM subjects from Norway and Pakistan. Eighteen immigrant Pakistani and 21 Norwegian T2DM subjects (age 29-45, 49% men) were included. They underwent anthropometrical measurements including bioelectrical impedance analysis, CT scans measuring fatty infiltration in liver and adipose and muscle tissue compartments in mid-abdomen and thigh, a euglycemic clamp, and blood samples for serum insulin and plasma glucose, adipokines and inflammation markers. Adipose tissue distribution was similar in Norwegians and Pakistanis. Pakistanis, but not Norwegians, showed a negative correlation between insulin sensitivity and visceral adipose tissue (VAT, $r_s = -0.704$, $p = 0.003$). Subcutaneous adipose tissue (SAT) correlated to leptin in both Pakistanis and Norwegians ($r_s = 0.88$, $p < 0.001$ and 0.67, $p = 0.001$). SAT also correlated to C-reactive protein (CRP) in the Pakistanis only ($r_s = 0.55$, $p = 0.03$), and superficial SAT to Interleukin-1 receptor antagonist (IL-1RA) in Norwegians only ($r_s = 0.47$, $p = 0.04$). In conclusion, despite similar adipose tissue distribution in the two groups Pakistanis were more insulin resistant, with a negative correlation of VAT to insulin sensitivity, not present in Norwegians. The correlation of adipose tissue to Leptin, CRP and IL-1RA showed ethnic differences.

Key words: Anthropometry; C-reactive protein; Computed tomography; Immigrants; Insulin resistance**;** Interleukin 1-receptor antagonist; Leptin; South Asia; Subcutaneous adipose tissue; Visceral adipose tissue.

Introduction

Immigrants from South Asia to Western countries show increased insulin resistance [1] and a high prevalence of type 2 diabetes mellitus (T2DM) [2;3]. The association between insulin resistance and obesity is well established. However, several reports indicate that commonly used methods of anthropometric measurements, like the body mass index (BMI), may be inadequate as indicators of metabolic risk in South Asians, who seem to develop insulin resistance and T2DM at levels of BMI that are considered low risk in the Western population [4;5].

Body composition can be measured using dual x-ray absorptiometry (DXA) or bioelectrical impedance analysis (BIA). However, these methods discriminate poorly between the various abdominal adipose tissue compartments such as subcutaneous (SAT) and visceral adipose tissue (VAT). Using computed tomography (CT) or magnetic resonance imaging (MRI), the adipose tissue compartments can be measured more accurately [6]. Previous studies have indicated that abdominal SAT and VAT are differently related to metabolic risk [7], and that VAT, and possibly deep subcutaneous adipose tissue (DSAT), is closely related to insulin resistance [8]. Increased fatty infiltration in the liver is also associated with overall insulin resistance [9].

Obesity and insulin resistance are accompanied by low-grade systemic inflammation with increased plasma levels of inflammatory markers, in part released from adipose tissue. Recent studies have suggested that the various adipose tissue compartments may differ in their inflammatory potential [10-12] in addition to their differing relation to insulin resistance.

The aim of this study was to investigate i) possible differences in the abdominal adipose tissue distribution between T2DM subjects from Norway and Pakistan, ii) whether ethnic differences in insulin resistance could be explained by differences in body composition, and iii) whether possible ethnic differences in insulin resistance and body composition could be related to plasma levels of inflammatory markers.

Materials and Methods

Ethics

The study was approved by the Eastern Norway Regional Committee for Medical and Health Research Ethics, and conformed to the Helsinki declaration. Informed written consent was obtained from each participant prior to any study related procedure.

Patients

The study population has previously been described [13]. Briefly, 18 Pakistani and 21 Norwegian patients (49% men), aged 29 to 45 years, with confirmed T2DM, were recruited from two hospital out-patient clinics, at Lovisenberg Deaconess Hospital and Aker University Hospital in Oslo. Clinical characteristics of the two ethnic groups are shown in Table I.

Anthropometrical measurements

Height and weight were measured with participants wearing light clothing and no shoes. BMI was calculated as weight in kg/(height in m)^2. Waist and hip circumferences were assessed with a tape measure with spring scale to ensure equal traction at every measurement, measuring at mid point between the lowest rib margin and the iliac crest, and at the level of the major trochanter, respectively. BIA was performed on 17 of the Norwegian and 14 of the Pakistani patients, on a Tanita Body Composition Analyzer BC-418 (Tokyo, Japan), providing measurements of percentage total body fat (%TBF), body fat mass in kilograms (BFM) and fat free mass in kilograms (FFM). All subjects were fasting and had voided urine before measurements.

Computed tomography measurements

CT was performed using a CT Somatom (Erlangen, Germany) with the patient examined in a supine position, arms extended above the head. Three single axial scans were performed

without intra-venous contrast medium through i) the liver and the spleen, at the level of Th12, ii) the mid-abdomen, 10 cm above L4/L5 in men and 5 cm above L4/L5 in women (Figure IA) [14], and iii) the thighs, at mid-distance between the anterior-superior iliac spine and the upper margin of the patella. CT parameters were 120 kV, 100 mAs, and slice thickness 4 mm. The dicom images were analysed using Osirix v 3.2.4, 32 bit.

The fat content of the liver was based on attenuation values in Hounsfield Units (HU). The liver-spleen ratio (LS-ratio) was calculated based on the mean of three measurements in the liver (2 right lobe and 1 left lobe) and two measurements in the spleen with a region of interest (ROI) 80 mm^2 (Figure IB). In mid-abdomen, the circumferences for subcutaneous adipose tissue (SAT) were tracked for both superficial (SSAT) and deep subcutaneous adipose tissue (DSAT) compartments, divided by the superficial fascia [15]. The visceral adipose tissue (VAT) compartment was measured by tracking the inner abdominal circumference, and measuring pixels with fat highlighted between -30 and -190 HU (Figure IC and D). The right thigh was selected for measuring thigh SAT and muscle compartments (Figure IE and F).

Euglycemic clamp

The euglycemic clamp method has previously been described [13]. Briefly, the patients underwent a two-step euglycemic, hyperinsulinemic clamp, using a modified version of the method originally described by De Fronzo et al. [16]. Human insulin (Actrapid®, Novo Nordisk, Copenhagen, Denmark) 300 mU/mL and Glucose 200 mg/mL were infused through a teflon catheter in a vein at the left elbow of the patient. Insulin was diluted in NaCl 0.9 %, after having first added 2 mL of the patients own blood, to avoid insulin sticking to the walls of the bag. All blood samples were drawn from a teflon catheter in a vein at the right elbow, kept open by a slow infusion of NaCl 0.9 %. The right arm was kept at 37°C by a heating sleeve connected to a thermal control unit (Swetron AB, Veddestad, Sweden), to arterialise

blood samples. Two successive clamp steps of 40 and 400 mU/m^2/min of insulin were performed, with a minimum duration of 100 minutes, and at least 30 minutes of stable euglycemia for each clamp step. The body surface area was calculated using Mostellers equation [17]. Plasma glucose was measured every five minutes using a OneTouch Ultra glucose measuring device (LifeScan, Milpitas, CA), with control measurements every 30 minutes by the glucose oxidase method, on a Glucose Analyzer II (Beckman Instruments, Fullerton, CA). The glucose infusion rate (GIR) in µmol/m^2/min was calculated for both clamp steps. The Insulin Sensitivity Index (ISI) was expressed as the ratio of the GIR to the prevailing mean serum insulin levels at the end of each clamp step ([GIR/I] x 100). Data presented here are from the first step of the clamp. Two Norwegian and two Pakistani patients did not attain euglycemia during the first step of the clamp, and were excluded from this part of the clamp analyses. After excluding these patients, there were 46% men in the first step of the clamp.

Blood samples

Fasting plasma glucose was measured by the glucose oxidase method on a Glucose Analyzer II (Beckman Instruments). Serum insulin was analysed using the radioimmunoassay (RIA) kit, formerly from Linco Research, presently available from Millipore Corp. (Billerica, MA). Plasma levels of adiponectin and leptin were analysed using RIA kits from Millipore Corp. (Billerica, MA),(also formerly from Linco Research). Plasma high sensitive C-reactive protein (hsCRP) was measured using a DuoSet ELISA kit from R&D Systems (Minneapolis, MN). Plasma interleukin-1 receptor antagonist (IL-1RA) was measured using CytoSet from Invitrogen Corporation (Carlsbad, CA), with streptavidin-horseradish peroxydase from R&D Systems. Plasma measurement of interleukin-6 (IL-6) was performed using a High Sensitivity ELISA kit from Abcam plc. (Cambridge, UK)

Statistical analyses

Data are presented as mean ± SD or median [inter-quartile range] unless otherwise specified. We analysed non-normally distributed data using non-parametric methods, or log-transformed, as appropriate. Student's *t* tests or Mann Whitney U tests were used for comparison of continuous variables between groups. For comparison of categorical data between patient groups, the Chi square test for independence was used. Spearman's correlation coefficient (r_s) was used. Multiple linear regression analyses were performed, with log-transformation of parameters when needed, to ensure no violation of the assumptions of normality, linearity and homoscedasticity. A two-sided p-value <0.05 was regarded as significant, but owing to the large number of comparisons, particular attention should be directed towards analyses where p-values are <0.01. Statistical analyses were performed with SPSS 19.0 for windows (SPSS Inc., Chicago, IL).

Results

Adipose tissue distribution

The Norwegians had higher weight, BMI, BFM and FFM than the Pakistanis, but there was no significant difference in the % TBF (Table I).

Liver fat infiltration and abdominal and thigh adipose tissue compartments did not differ between the two ethnic groups (Table II). The relative size of VAT as determined by the VAT/SAT ratio, was significantly smaller in women than in men (0.49 [0.35] vs. 1.26 [0.84], p<0.001), but again, there was no ethnic difference. For women, abdominal and thigh muscle compartments were significantly larger in the Norwegian group, while for men, there was only a tendency towards larger abdominal muscle compartment in the Norwegians (Table II). SAT was found to correlate well to several anthropometrical markers of obesity, such as BMI, waist circumference and %TBF in both groups. VAT on the other hand, correlated less well to

BMI and waist circumference, with particularly weak correlations in the Norwegian group. Moreover, VAT was paradoxically, negatively correlated to %TBF in the Norwegian group with no significant correlation in the Pakistani group (Table III).

Insulin sensitivity

Insulin sensitivity (ISI_{40}) was negatively correlated to VAT and positively correlated to liver attenuation and LS-ratio, but not to SAT, in the total patient group. However, when analysing each ethnic group separately, the negative association with VAT and liver fat was present in the Pakistani group while it was not significant in the Norwegian group (Table IV). Using multiple regression analysis examining predictors of insulin sensitivity, we found that 38.6 % of $LogISI_{40}$ variation was explained by the combination of VAT ($p<0.001$), sex ($p=0.015$) and ethnicity ($p=0.046$), (model significance: $p=0.002$). The total DSAT+VAT compartment was also significantly correlated to ISI_{40}, and again, this did reach statistical significance in the Pakistani but not in the Norwegian group (Table IV). In line with this, the correlation between DSAT+VAT and ISI_{40} became stronger ($p=0.001$) when adjusting for ethnicity ($p=0.023$) in a multiple regression analysis with $logISI_{40}$ as dependent variable (model significance: $p=0.003$, $R^2=0.31$).

Adipokines and inflammatory markers

Plasma levels of leptin, CRP and IL-1RA showed significant correlations with adipose tissue compartments, while no association was shown for adiponectin or IL-6 (Table V). Plasma levels of leptin were highly positively correlated to abdominal and thigh SAT in both groups, with the strongest correlations in the Pakistani group. In fact, in a multiple regression analysis exploring predictors of plasma leptin, we found that 77.8 % of the variation in leptin was explained by SSAT ($p<0.001$), sex ($p<0.001$) and ethnicity ($p=0.046$) (model significance:

p<0.001). Moreover, CRP was positively correlated to abdominal SAT in the Pakistani group, and IL-1RA to abdominal and thigh SAT in Norwegian group only.

Discussion

In this study, we report both ethnic similarities and differences in the distribution of adipose and muscle tissue, as well as their relations to insulin sensitivity and inflammatory markers. The Norwegian and Pakistani groups showed no significant differences in the areas of adipose tissue compartments measured by CT in abdomen or thigh. However, we observed ethnic differences in how abdominal adipose tissue compartments such as SSAT, DSAT and VAT related to insulin sensitivity and inflammatory markers. VAT appeared to be more metabolically active in the Pakistani than in the Norwegian group, displaying strong negative correlations to insulin sensitivity. In addition, an association between CRP and abdominal SAT was only seen in the Pakistani group, suggesting a stronger link between SAT and inflammation in Pakistanis with T2DM as compared with the Norwegians.

The significant positive correlations of CRP to SAT compartments, but not to VAT in our Pakistani patients are at variance with some previous reports [11;12]. This may be due to the higher BMI, %TBF and HbA1c values in both our ethnic groups than those reported in other studies, making comparisons difficult. In a cohort of severely obese subjects, CRP was found to be a non-specific marker of obesity, and not related to specific adipose tissue compartments or metabolic dysregulation like the metabolic syndrome, T2DM or non-alcoholic steato-hepatitis [18]. SAT in our study correlated better than VAT to general measurements of obesity, thus possibly explaining these findings in our Pakistani group. Nonetheless, CRP is a reliable down-stream marker of inflammation, and the positive correlation of CRP to SAT in the Pakistani group suggests a more inflammatory potential in SAT in the Pakistani group.

We found similar abdominal adipose tissue distribution in the Pakistani and Norwegian groups. Recently, Lear and co-workers demonstrated that healthy South Asian men and women had higher levels of both SAT and VAT than a similar group of subjects of European ancestry [19]. The same authors have however previously shown that the ethnic differences in VAT between the South Asians and Europeans in this cohort were only evident when waist circumference was lower than 105 cm [20]. Other authors have found no ethnic differences in adipose tissue distribution, at least in men [21;22]. In our study population the mean waist circumferences were wider than 105 cm in both ethnic groups, and BMI was higher than in the studies mentioned (Table I). The higher degree of adiposity in our study, where the subjects also had established T2DM, could therefore have attenuated ethnic differences in VAT, and possibly also in SAT.

Leptin levels were strongly and positively correlated to areas of SAT in both ethnic groups, as expected. This was also seen for IL-1RA in the Norwegian group. Leptin has been shown to induce monocyte expression of IL-1RA [23], and increased levels of IL-1RA are correlated to the hyperleptinemia of obesity [24]. IL-1RA has been shown to correlate to SAT and especially to VAT [25]. Increasing IL-1RA levels have also been associated with the development of T2DM [26]. In our patients, IL-1RA, like leptin, correlated positively to SAT in abdomen and thigh in the Norwegian, but not the Pakistani group. There was no significant correlation of IL-1RA to VAT. Our results may indicate that IL-1RA is a more important factor in the Norwegian than in the Pakistani group. However, due to relatively small numbers included, conclusions should be drawn with care..

In our Norwegian group, the adipose tissue compartments seemed to be less important for determining insulin sensitivity. The mean age at diagnosis of T2DM in subjects from South

Asia living in Norway has previously been found to be more than 10 years lower than in ethnic Norwegians [27]. We might speculate that by having selected young Norwegian T2DM subjects these could have a stronger genetic predisposition to diabetes. Obesity might therefore be of somewhat less importance for the development of T2DM in the Norwegian group. The Pakistani subjects on the other hand, were closer in age to the average Pakistani T2DM patient in Norway. The negative correlation between insulin sensitivity and VAT, seen only in the Pakistani group, may also reflect genuine ethnic differences in the composition and metabolic character of VAT, with VAT being more metabolically active in Pakistanis than in Norwegians.

The strengths of this study include employing the two-step euglycemic, hyperinsulinemic clamp for measuring insulin sensitivity, and having both CT and BIA measurements of body composition. The groups we have studied are of restricted numbers, and this constitutes a limitation, increasing the risk of not being able to detect small but possibly important differences. Furthermore, we report many statistical tests without correction for multiple testing, with the risk of reporting p-values <0.05 by chance. We therefore focused on the most robust statistical findings in our discussion and conclusions, and have taken care to give the exact p-values throughout the manuscript, to facilitate the possibility for the reader to judge. By recruiting patients from hospital out-patient clinics only, there may be a selection bias towards including subjects with T2DM that is not easily treated in general practice, and the findings should be interpreted in this context.

In conclusion, we report that in spite of similar adipose tissue distribution in the Norwegian and Pakistani groups, there is evidence of ethnic differences in the importance of adipose tissue distribution for insulin sensitivity, where visceral adipose tissue seems to be especially

important in the metabolic dysregulation in Pakistanis. We have also found differences in the relationship between adipose tissue distribution and some adipokines and markers of inflammation, although further investigation in this field is needed.

Conflicts of interests statement

There are no potential conflicts of interest relating to this publication.

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1 **Table I: Clinical characteristics of T2DM patients according to ethnic group**

	Norwegians n = 21	**Pakistanis** n = 18	**p**
Males n (%)	10 (48%)	9 (50%)	
Age (years)*	42 [15]	41 [16]	0.865
Years with diabetes*	5 [16]	9 [17]	**0.021**
Diabetes treatment n (%):			
Lifestyle ± OAD / Insulin ± OAD	11 (52%) / 10 (48%)	5 (28%) / 13 (72%)	0.119
Weight (kg)	106.8 [49.3]	90.1 [86.9]	**0.009**
BMI (kg/m^2)*	37.2 [15.0]	30.9 [35.2]	**0.008**
Waist circumference (cm)	114.3 [50.0]	106.5 [59.5]	0.102
Waist-Hip ratio	1.00 [0.34]	1.01 [0.36]	0.575
Total body fat (%)	36.9 [29.9]	34.2 [27.4]	0.395
Body fat mass (kg)	39.5 [43.7]	28.0 [25.5]	**0.005**
Lean body mass (kg)	67.1 [39.4]	54.2 [34.2]	**0.007**
HbA1c (%)*	7.3 [5.6]	8.7 [6.5]	**0.022**

2
3 **Table I:** Data are shown as mean [range] with comparisons using Student's t test. * For parameters with non-normal
4 distribution median [range] are given, with comparisons using the Mann-Whitney U test. Diabetes treatment is compared using
5 Chi square test for independence. OAD: oral anti-diabetic treatment. $p < 0.05$ in bold.
6

7 **Table II: Adipose tissue and muscle distribution in Norwegians and Pakistanis with T2DM**

	Women			**Men**		
	Nor n=11	**Pak** n=9	**p**	**Nor** n=10	**Pak** n=9	**p**
SSAT (cm^2)[a]*	270 [86]	237 [160]	0.36	155 [75]	125 [117]	0.18
DSAT (cm^2)[a]*	161 [122]	125 [89]	0.59	105 [125]	74 [39]	0.09
VAT (cm^2)[b]*	217 ± 71	210 ± 59	0.84	282 ± 49	264 ± 73	0.53
VAT/SAT ratio[b]*	0.52 ± 0.20	0.57 ± 0.23	0.59	1.21 ± 0.74	1.56 ± 0.94	0.39
SAT thigh (cm^2)[a]	192 [90]	169[73]	0.27	105 [52]	95 [71]	0.29
Liver attenuation (HU)[b]	44.9 ± 23.0	50.8 ± 11.5	0.49	52.7 ± 9.0	47.8 ± 12.4	0.33
LS ratio[b]	0.88 ± 0.45	0.98 ± 0.21	0.57	0.98 ± 0.17	0.88 ± 0.22	0.31
Abdominal muscle area (cm^2)[b]*	188 ± 28	157 ± 21	**0.02**	220 ± 12	197 ± 31	0.07
Thigh muscle area (cm^2)[b]	156 ± 25	130 ± 21	**0.03**	194 ± 19	182 ± 38	0.39

8 **Table II:** [a] Data are shown as median [IQR], two-sided p from Mann Whitney U-test. [b] Data are shown as
9 mean ± SD, two-sided p from Student's *t* test. Nor = Norwegians, Pak = Pakistanis, SSAT = superficial
10 subcutaneous adipose tissue, DSAT = deep subcutaneous adipose tissue, VAT = visceral adipose tissue,
11 SAT thigh = subcutaneous adipose tissue in right thigh, Liver attenuation in Hounsfield units (HU), LS ratio
12 = Liver-Spleen ratio. * Pakistani women: n=8

13 **Table III: Correlations of adipose tissue compartments to anthropometrical**
14 **measurements**

		SAT		**VAT**	
		Nor	**Pak**	**Nor**	**Pak**
BMI	r_s	**0.87**	**0.74**	-0.02	0.46
	p	**< 0.001**	**0.001**	0.94	0.06
Waist circumference	r_s	**0.59**	**0.72**	0.23	0.45
	p	**0.006**	**0.001**	0.34	0.07
% TBF	r_s	**0.72**	**0.92**	**-0.57**	0.05
	p	**0.001**	**< 0.001**	**0.017**	0.88

15

16 **Table III:** Spearman's correlation coefficients (r_s) for SAT (subcutaneous adipose
17 tissue) and VAT (visceral adipose tissue). BMI (body mass index), %TBF (percentage
18 total body fat). Nor = Norwegians, Pak = Pakistanis.

19

Table IV: Correlations of adipose tissue compartments to the Insulin Sensitivity Index in Norwegian and Pakistani patients with T2DM

		ISI_{40}		
		Tot n=34	**Nor** n=19	**Pak** n=15
SSAT	r_s	-0.253	-0.308	-0.339
	p	0.150	0.199	0.216
DSAT	r_s	-0.068	-0.160	-0.141
	p	0.702	0.514	0.615
VAT	r_s	**-0.424**	-0.249	**-0.704**
	p	**0.013**	0.304	**0.003**
DSAT+VAT	r_s	**-0.432**	-0.338	**-0.781**
		0.011	0.157	**0.001**
Liver Att.*	r_s	**0.404**	0.319	0.427
	p	**0.016**	0.182	0.099
LS-ratio *	r_s	**0.401**	0.329	0.389
	p	**0.017**	0.169	0.137

20 **Table IV:** Spearman's correlation coefficients (r_s) for SSAT (superficial subcutaneous
21 adipose tissue), DSAT (deep subcutaneous adipose tissue), VAT (visceral adipose
22 tissue), and Liver Att. (Liver attenuation). Correlation coefficients with p-values < 0.05
23 are in bold. * Total n=35, Pakistani n=16. Tot = total, Nor = Norwegians, Pak =
24 Pakistanis
25

Table V: Correlations of adipose and muscular tissue compartments to markers of inflammation

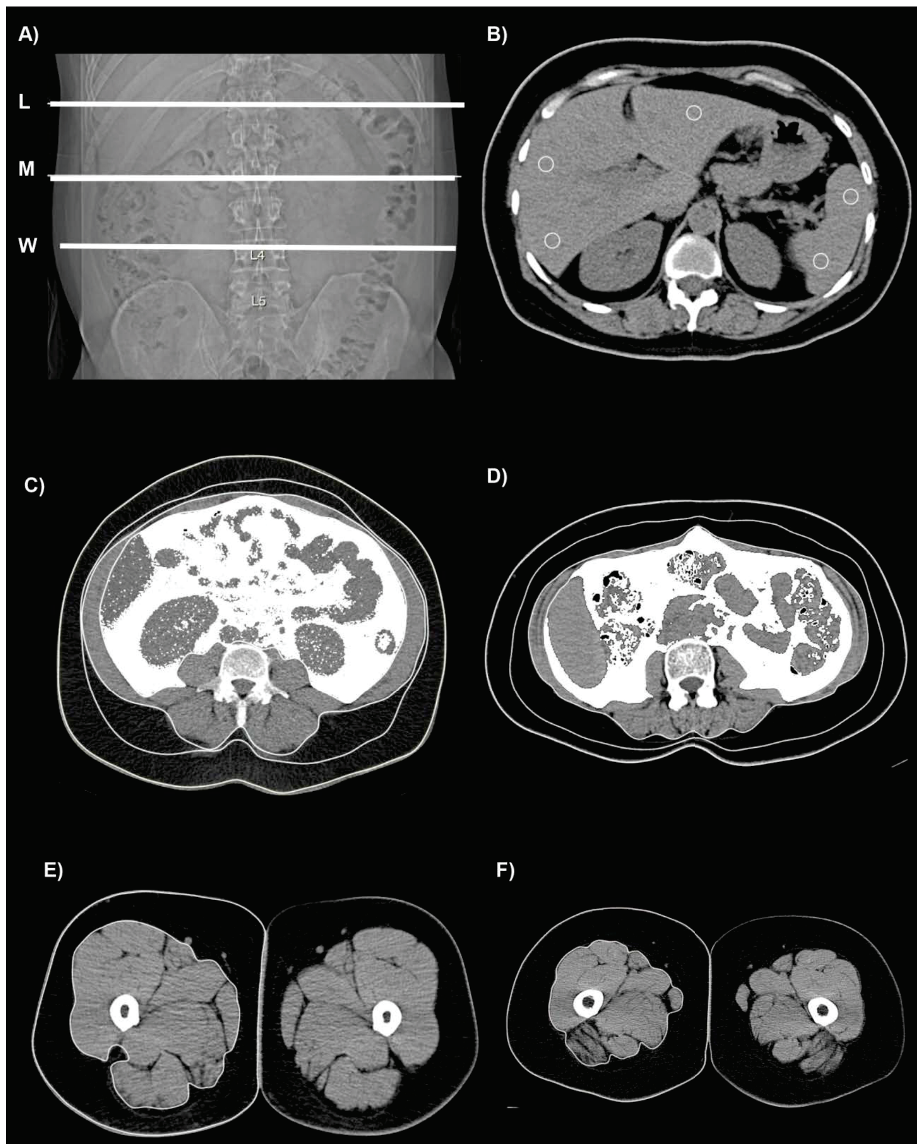
		Adiponectin		**Leptin**		**CRP**		**IL-6**		**IL-1RA**	
		Nor (n=21)	Pak (n=16)	Nor (n=21)	Pak (n=16)	Nor (n=20)	Pak (n=16)	Nor (n=20)	**Pak** (n=16)	**Nor** (n=20)	**Pak** (n=16)
SSAT	r_s	-0.18	0.27	**0.76**	**0.86**	-0.28	0.50	0.07	0.30	**0.47**	0.25
	p	0.44	0.29	**<0.001**	**<0.001**	0.23	0.05	0.76	0.26	**0.04**	0.35
DSAT	r_s	0.04	-0.10	0.44	**0.68**	0.00	**0.56**	-0.23	0.25	-0.35	0.26
	p	0.85	0.69	0.05	**0.003**	0.99	**0.03**	0.33	0.35	0.13	0.33
SAT	r_s	-0.11	0.19	**0.67**	**0.88**	-0.18	**0.55**	-0.08	0.37	0.43	0.24
	p	0.63	0.49	**0.001**	**<0.001**	0.44	**0.03**	0.73	0.16	0.06	0.37
VAT	r_s	-0.38	-0.06	-0.12	-0.03	0.10	-0.212	0.15	-0.33	0.15	0.08
	p	0.09	0.82	0.60	0.90	0.68	0.43	0.52	0.22	0.53	0.76
VAT/SAT	r_s	-0.11	-0.21	**-0.60**	**-0.77**	0.02	**-0.60**	0.05	-0.45	-0.29	-0.16
	p	0.63	0.42	**0.004**	**<0.001**	0.94	**0.01**	0.85	0.08	0.21	0.56
SAT Thigh*	r_s	0.04	0.23	**0.56**	**0.88**	-0.08	0.24	0.21	-0.01	**0.49**	0.15
	p	0.88	0.37	**0.01**	**<0.001**	0.74	0.36	0.38	0.96	**0.03**	0.56
Liver att.*	r_s	-0.11	0.38	-0.25	0.16	0.30	0.20	0.23	0.45	-0.38	-0.35
	p	0.65	0.12	0.28	0.52	0.20	0.44	0.32	0.07	0.10	0.17
Abdo Musc.	r_s	-0.01	-0.09	-0.39	-0.23	-0.06	-0.01	-0.14	-0.10	-0.28	0.44
	p	0.95	0.73	0.08	0.38	0.81	0.98	0.56	0.71	0.24	0.09
Thigh Musc.*	r_s	0.17	-0.40	-0.43	-0.33	0.13	-0.11	-0.31	-0.24	-0.26	0.39
	p	0.46	0.11	0.05	0.18	0.59	0.67	0.18	0.35	0.26	0.12

26 **Table V:** Spearman's correlation coefficients (r_s) between superficial subcutaneous adipose tissue (SSAT), deep subcutaneous adipose tissue
27 (DSAT), total subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), visceral-to-subcutaneous adipose tissue ratio (VAT/SAT),

28 subcutaneous adipose tissue in thigh (SAT Thigh), liver attenuation (Liver Att.), abdominal musculature (Abdo Musc.), thigh musculature (Thigh
29 Musc.) and various adipokines and markers of inflammation. CRP: C-reactive protein, IL-6: Interleukin-6, IL-1RA: Interleukin-1 receptor
30 antagonist . Correlation coefficients with p-values < 0.05 in bold. * For Pakistani patients n=17.

31 **Figure I: Computed tomography measurements in Norwegian and Pakistani**

32 **patients with T2DM**



33

34 **Figure I**: **A)** Abdominal scanogram marked with the levels of the two axial CT scans,

35 with the most cranial through the liver and spleen (L), and the caudal scan 10 cm above

36 L4/L5 in men (M) or 5 cm above L4/L5 in women (W). **B)** Axial CT scan showing

37 three regions of interest (ROI) in the liver and two in the spleen, for measurement of

38 attenuation in Hounsfield Units. **C)** and **D)** Axial mid-abdominal CT-scans showing

39 tracking of fat and muscle compartments and highlighting of VAT in a Norwegian
40 woman (C) and Pakistani woman (D) with large and small abdominal muscle areas
41 respectively. **E)** and **F)** CT-scans through thighs in a Norwegian (E) and a Pakistani (F)
42 woman showing the tracking of fat and muscle compartments.
43

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130

III

Characteristics of Glucose Metabolism in Nordic and South Asian Subjects with Type 2 Diabetes

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Abstract

Background: Insulin resistance and type 2 diabetes are more prevalent in people of South Asian ethnicity than in people of Western European origin. To investigate the source of these differences, we compared insulin sensitivity, insulin secretion, glucose and lipid metabolism in South Asian and Nordic subjects with type 2 diabetes.

Methods: Forty-three Nordic and 19 South Asian subjects with type 2 diabetes were examined with intra-venous glucose tolerance test, euglycemic clamp including measurement of endogenous glucose production, indirect calorimetry measuring glucose and lipid oxidation, and dual x-ray absorptiometry measuring body composition.

Results: Despite younger mean ± SD age (49.7±9.4 vs 58.3±8.3 years, *p = 0.001*), subjects of South Asian ethnicity had the same diabetes duration (9.3±5.5 vs 9.6±7.0 years, *p = 0.86*), significantly higher median [inter-quartile range] HbA_{1c} (8.5 [1.6] vs 7.3 [1.6] %, *p = 0.024*) and lower BMI (28.7±4.0 vs 33.2±4.7 kg/m^2, $p<0.001$). The South Asian group exhibited significantly higher basal endogenous glucose production (19.1 [9.1] vs 14.4 [6.8] µmol/kgFFM·min, p = *0.003*). There were no significant differences between the groups in total glucose disposal (39.1±20.4 vs 39.2±17.6 µmol/kgFFM·min, *p = 0.99*) or first phase insulin secretion ($AUC_{0-8\ min}$: 220 [302] vs 124 [275] pM, p = *0.35*). In South Asian subjects there was a tendency towards positive correlations between endogenous glucose production and resting and clamp energy expenditure.

Conclusions: Subjects of South Asian ethnicity with type 2 diabetes, despite being younger and leaner, had higher basal endogenous glucose production, indicating higher hepatic insulin resistance, and a trend towards higher use of carbohydrates as fasting energy substrate compared to Nordic subjects. These findings may contribute to the understanding of the observed differences in prevalence of type 2 diabetes between the ethnic groups.

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Introduction

The prevalence of type 2 diabetes (T2D) varies between different ethnic groups, and is known to be high in South Asians (SA) in their countries of origin, particularly in urban areas, but also after migration to Western countries [1]. Several theories as to why SA are especially prone to insulin resistance and T2D have been proposed. Among these, the adipose tissue overflow hypothesis proposes that SA have smaller superficial subcutaneous adipose tissue compartments for fat storage compared to Western Europeans. Hence, in situations of energy excess, fat is deposited in the more metabolically active deep subcutaneous and visceral adipose tissue compartments [2]. The metabolic inflexibility theory proposes that the normal switch in energy substrate between high lipid oxidation in the fasting state and high glucose oxidation in the post-prandial state is impaired in T2D. This results in accumulation of intramuscular lipids and increased plasma glucose values [3]. A third suggested mechanism involves the theory that SA have lower resting energy expenditure (REE) than Western Europeans, and therefore are more prone to obesity and insulin resistance. This theory is disputed, where some argue that the lower REE is due to differences in body composition and not ethnicity [4,5].

Although insulin resistance and T2D have been much studied in the SA population during recent years, few studies have used gold standard methods for the measurement of insulin resistance, and to our knowledge, no previous studies have reported endogenous glucose production (EGP) in SA T2D subjects.

In order to gain insights into possible explanations for the differences in prevalence of T2D between the two populations, we analysed baseline data from a vitamin D intervention trial in subjects with T2D and Nordic (NOR) or SA origin, all living in Oslo, Norway. Insulin sensitivity, glucose and fat oxidation, and insulin secretion was measured. The subjects all had serum levels of 25-hydroxyvitamin D ≤50 nM.

The primary aims of this study were to: 1) Explore possible differences in fasting and clamp hyperinsulinemic glucose metabolism, 2) Explore possible differences in insulin secretion, 3) Explore possible differences in energy expenditure, and 4) Assess possible associations between insulin sensitivity and -secretion, and vitamin D status.

Methods

Ethics

All participants gave informed written consent prior to any study related procedure. The study was approved by the South-Eastern Norway Regional Committee for Medical and Health Research Ethics, and conformed to the Declaration of Helsinki.

Subjects

Sixty-two patients with T2D and 25-hydroxyvitamin D $\leq$50 nM, were recruited from our out-patient clinic, from general practice, from posters in the hospital lobby and in pharmacies, and from advertisements in local newspapers. Men and women with T2D, from the Oslo area in Norway, above 18 years of age, of Nordic or South Asian origin (born in a South Asian country and/or with both parents of South Asian origin) were eligible, regardless of type of anti-diabetic treatment. Full inclusion and exclusion criteria are displayed in Table 1. In total, 190 patients were screened. Sixty-two patients met with the inclusion criteria and were recruited, and 61 patients underwent initial intra-venous glucose tolerance tests (IVGTT) and clamp procedures. The Nordic group consisted of 40 Norwegians, 2 Danes and 1 Swede. The South Asian group consisted of 11 Pakistanis, 7 Sri Lankans (all Tamil) and 1 Indian. All but one of the South Asian participants were first generation immigrants. One Norwegian patient was excluded due to severe difficulties in getting intravenous access. Table 2 shows important characteristics of the two ethnic groups.

Anthropometrics

Height to the nearest 0.1 cm and weight to the nearest 0.1 kg were measured with participants wearing light clothing and no shoes. Waist circumference was assessed with a flexible tape measure with spring scale to ensure equal traction at every measurement, measuring at mid-point between the lowest rib margin and the iliac crest. The body surface area was calculated using Mostellers equation [6]. Fat mass (FM) in kg, percentage total body fat (%TBF), percentage truncal fat (% truncal fat) and fat free body mass (FFM) in kg were measured by dual x-ray absorptiometry (DXA) on a Lunar Prodigy from GE Healthcare.

IVGTT and Euglycemic Clamp

To enhance comparability of examinations, all patients were asked to stop oral antidiabetic drugs for two days, and insulin for at least 12 hours prior to examination. Patients were also asked to refrain from strenuous physical exercise and alcohol intake during these two days, and to arrive fasting for at least 10 hours, from the night before the examination.

A teflon catheter was placed in a vein at each elbow. All infusions were given in one vein, and all blood samples were drawn from the other vein, which was kept open by a slow infusion of NaCl 0.9%. The arm where blood samples were taken was kept at 37°C by a heating sleeve connected to a thermal control unit (Swetron AB, Veddestad, Sweden), to arterialize blood samples.

We performed an IVGTT followed by a euglycemic, hyperinsulinemic clamp, with estimation of endogenous glucose production (EGP) using the stable isotope dilution method. A primed (170 mg) continuous (1.7 mg/min) infusion of [6,6-$^2H_2$] glucose (Cambridge Isotope Laboratories, Inc., Andover, MA) was maintained throughout the experiment. After a 2-h tracer equilibration period, the IVGTT was performed, with a $<$1-minute intravenous bolus injection of glucose 500 mg/mL, 0.3 g/kg body weight. Blood samples were drawn for plasma glucose concentration as well as serum insulin and C-peptide determination at −2, 0, 2, 4, 6, 8, 10, 15 and 30 minutes after glucose bolus injection. Immediately following the IVGTT, a euglycemic, hyperinsulinemic clamp was performed using a modification of the method originally described by De Fronzo et al [7]. Human insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) was diluted in 500 mL NaCl 0.9%, to 300 mU/mL, after having first added 2 mL of the patients own blood, to avoid insulin sticking to the walls of the bag, and 10 mmol KCl. Insulin was infused at a rate of 80 mU/m^2·min, after an initial bolus and 10 minute priming infusion, determined by the patients pre-clamp plasma glucose. The infusion was maintained for 2 ½ hours or more, until at least 30 minutes of stable euglycemia was obtained. When plasma glucose reached euglycemia, a variable infusion of glucose 200 mg/mL enriched with 8 mg/g glucose of [6,6-$^2H_2$]-glucose was continually adjusted to maintain euglycemia.

Plasma glucose was regularly measured on a Presicion Xceed glucometer (Abbott Laboratories. Abbott Park, IL), with five-minute intervals when the patient approached euglycemia. Control measurements at least every 30 minutes were performed on a Y.S.I 2300 STAT analyzer (Yellow Springs Instruments Inc, Yellow Springs, OH). At the end of the clamp, three measurements of serum insulin and fluoride/oxalate-plasma for analysis of [6,6-$^2H_2$]-glucose were taken at ten-minute intervals. The glucose infusion rate (GIR) in μmol/kg FFM·min was established.

IVGTT Calculations of Insulin Secretion

The Acute Insulin Response to glucose (AIRg) was calculated as the incremental area under the curve (AUC) for insulin from time 0–8 minutes and 0–30 minutes.

Endogenous Glucose Production Calculations

Calculations of endogenous glucose production (EGP) at the end of the basal equilibration period and during clamp euglycemia were performed. Both were steady state for plasma glucose, with only relatively small changes in glucose concentration and tracer enrichment over time. Thus, steady state equations, where rate of appearance equals rate of disappearance, have been applied for the calculation of both EGP and total glucose disposal (TGD) [8,9]. The EGP in the basal state was calculated as follows: $EGP_{basal} = I((E_i/E_{p(basal)}) -1)$, where I is the rate of [6,6-$^2H_2$]-glucose infusion (μmol/m^2·min), E_i is the enrichment of the tracer infusion in moles percent excess (mpe) and $E_{p(basal)}$ is the mean [6,6-$^2H_2$]-glucose enrichment in plasma (mpe) at the end of the basal stabilisation period.

At the end of the euglycemic clamp, TGD was calculated as follows: $TGD = ((I \cdot E_i + GIR \cdot E_m)/E_{p(clamp)}) - I$, where GIR is the exogenous glucose infusion rate (μmol/m^2·min), E_m is the [6,6-$^2H_2$]-glucose enrichment (mpe) in the infused glucose, and $E_{p(clamp)}$ is the mean [6,6-$^2H_2$]-glucose enrichment (mpe) in the plasma samples taken during the last 30 minutes of the clamp euglycemia. The EGP during clamp euglycemia, $EGP_{clamp} = TGD - GIR$. Between subject and within subject coefficients of variation for plasma glucose levels in clamp steady state were 10.0% and 4.6% respectively.

Table 1. Inclusion and exclusion criteria.

Inclusion criteria:
Vitamin D deficiency defined as 25-hydroxyvitamin D <50 nM
Patients with type 2 diabetes (negative anti-GAD and anti-IA2), including drug naïve subjects, subjects using oral anti-diabetic medication and subjects on insulin treatment
HbA_{1c} <11% (97 mmol/mol) at inclusion
Men and women ≥18 years
Nordic or South Asian ethnicity (from Pakistan, India, Bangladesh or Sri Lanka)
Antihypertensive medication, lipid lowering drugs, oral contraceptives, hormone replacement therapy, multivitamin supplements and nutritional supplements are allowed
Exclusion criteria:
Systolic Blood Pressure ≥160 mmHg or Diastolic Blood Pressure ≥90 mmHg at inclusion
Significant renal disease or chronic renal impairment, GFR<30 mL/min
Significant liver disease or ASAT or ALAT >3× upper limit of normal
Malignancy during the last five years
Hypercalcemia at inclusion or a history of kidney stone disease
Pregnant or breastfeeding women
Chronic inflammatory disease in active phase or long term (>2 weeks) use of systemic corticosteroids last 3 months
Cardiovascular disease, defined as myocardial infarction, unstable angina pectoris or stroke, during the last 6 months prior to inclusion
Anemia defined as hemoglobin below current reference limits
BMI >45 kg/m^2 or bariatric surgery performed during the last five years
Drug or alcohol abuse
Mental condition (psychiatric or organic cerebral disease) rendering the subject unable to understand the nature, scope and possible consequences of the study
Any medical condition that in the judgment of the investigator would jeopardize the subject's safety

Main inclusion and exclusion criteria. GAD: glutamic acid decarboxylase, IA2: protein tyrosine phosphatase, GFR: glomerular filtration rate, ASAT: aspartate amino transferase, ALAT: alanine amino transferase, BMI: body mass index.
doi:10.1371/journal.pone.0083983.t001

Table 2. Description of patients.

	NOR	SA	
	n = 43	**n = 19**	***p***
Sex, males n (%)	28 (65.1%)	9 (47.4%)	*0.263* [b]
Age, years	58.3±8.3	49.7±9.4	***0.001***[a]
Age at diabetes debut, years	48.7±9.1	40.4±10.4	***0.002***[a]
Diabetes duration, years	9.6±7.0	9.3±5.4	*0.864*[a]
Diabetes medication, n (%):			*0.564*[b]
Lifestyle ± OAD or GLP-1	26 (60.5%)	10 (52.6%)	
Insulin ± OAD	17 (39.5%)	9 (47.4%)	
Diabetes complications, n (%):	20 (46.5%)	8 (42.1%)	*0.788*[b]
Cardiovascular disease	3 (7.0%)	2 (10.5%)	
Nephropathy/microalb.	8 (18.6%)	2 (10.5%)	
Other	17 (39.5%)	7 (36.8%)	

Data are presented as number (percentage) or as mean ± standard deviation. *p*-values from [a]Student's t-test or [b]Chi square test. OAD: oral antidiabetic agent, GLP-1: Glucagon-like peptid 1 analogue. Microalb.: microalbuminuria. Other complications include ophthalmopathy, neuropathy, diabetic foot, sexual dysfunction and periodontal disease. NOR = Nordic, SA = South Asians.
doi:10.1371/journal.pone.0083983.t002

Table 3. Anthropometrical and biochemical characteristics.

	NOR	SA	
	n = 43	**n = 19**	***p***
Height, cm	173.6±8.8	163.4±8.4	***<0.001***
Weight, kg	100.4 [15.0]	79.1 [15.0]	***<0.001***[a]
BMI, kg/m^2	33.2±4.7	28.7±4.0	***<0.001***
Waist circumference, cm	115.5 [18.0]	100 [10.6]	***<0.001***[a]
Waist/Height ratio	65.5±7.2	61.2±6.8	***0.033***
TBF, %	35.7±7.2	34.5±7.6	*0.56*
Truncal fat, %	40.5±5.7	40.1±6.6	*0.82*
FFM, kg	63.8±9.7	50.1±7.7	***<0.001***
FM, kg	33.1 [17.1]	25.0 [10.8]	***<0.001***[a]
Fasting plasma glucose, mM	9.1 [4.5]	10.7 [6.4]	*0.08*[a]
HbA_{1c}, %	7.3 [1.6]	8.5 [1.6]	***0.024***[a]
Fasting insulin, pM	85.5 [99.0]	68.0 [138.0]	*0.67*[a]
Fasting C-peptide, pM	1137 [785]	1012 [431]	***0.049***[a]
25(OH)vitamin D, nM	40.1±10.3	31.5±14.0	***0.009***

Data are presented as mean ± standard deviation or median [inter-quartile range]. NOR: Nordic, SA: South Asians, BMI: body mass index, TBF: total body fat, FFM: fat free mass, FM: fat mass, 25(OH)vitamin D: 25-hydroxyvitamin D. *p*-values are from Student's *t*-test.
[a] = Student's *t*-test after Log-transformation.
doi:10.1371/journal.pone.0083983.t003

Indirect Calorimetry

Indirect calorimetry was performed in 38 of the 43 NOR and 14 of the 19 SA patients, using a Jaeger Oxycon Pro (Erich Jaeger, Viasys Healthcare, Germany) computerized flow-through canopy gas analyzer system. After a 10-minute adaptation period, expired and inspired air was continuously sampled and analyzed for O_2 and CO_2 content during a 30 minute steady state period at the end of the basal tracer equilibration period and at the end of the euglycemic clamp. Whole body substrate oxidation was estimated from the mean values of VO_2 and VCO_2 measured, and from measurement of urinary nitrogen (urea). Average basal and insulin stimulated glucose and lipid oxidation rates were calculated using Frayn's equations [10]. Non-oxidative glucose metabolism was calculated as the difference between total body glucose disposal (as determined by the euglycemic clamp with tracer dilution method) and the rate of glucose oxidation (as determined by indirect calorimetry).

Blood Samples

Full blood glucose was measured by glucose oxidase method (YSI 2300, Yellow Springs, OH), and plasma glucose was calculated (full blood glucose×1.119). HbA_{1c} was measured by HPLC on a Tosoh G7 analyser (Tosoh Corp., Tokyo, Japan), serum insulin and C-peptide were measured using an immuno-fluorometric assay (DELFIA) from Perkin Elmer Life Sciences (Wallac Oy, Turku, Finland), 25-hydroxyvitamin D was measured on a radioimmunoassay (RIA) kit from DiaSorin (Stillwater, MN). [6,6-$^2H_2$]-glucose was measured by LC-MS/MS, via turbulent flow chromatography (Cohesive technologies RXT1, Franklin, MA) combined with tandem mass spectrometry (Sciex API3000, Applied Biosystems, Foster City, CA) as previously described [11], at the Clinical Metabolomics Core Facility, (Rigshospitalet, Copenhagen, Denmark). Urinary urea was measured by enzymatic-kinetic UV assay on a Roche Modular P analyser.

Statistical Analysis

Data are presented as mean ± standard deviation or median [inter-quartile range] unless otherwise specified. We analyzed non-normally distributed data log-transformed, or using non-parametric methods, as appropriate. Student's *t* tests or Mann Whitney U tests were used for comparison of continuous variables between groups, and paired samples *t*-tests were used for within groups analysis of change. For comparison of categorical data between patient groups, the Chi square test for independence was used. Spearman's correlation coefficients (r_s) were used. One–way between-groups ANCOVA was performed, with preliminary checks to ensure no violation of the assumptions of normality, linearity, homogeneity of variances and homogeneity of regression slopes. Multiple linear regression analyses were performed, with log-transformation of parameters when needed, to ensure no violation of the assumptions of normality, linearity and homoscedasticity. In regression analyses NOR = 1 and SA = 2. A two-sided p-value <0.05 was deemed significant, and uncorrected p-values are presented. Bonferroni-Holm corrections were performed, showing that p-values <0.01 remained <0.05 after correction. Statistical analyses were performed with SPSS 19.0 for windows (SPSS Inc., Chicago, IL).

Results

General Description

Anthropometric and biochemical characteristics by ethnic group are presented in Table 3. The SA subjects were significantly shorter and leaner than the NOR subjects, but had a higher median HbA_{1c}, whereas median fasting C-peptide was significantly higher in the NOR group. Despite a higher waist circumference and waist-to-height ratio in the NOR subjects, the SA still had similar percentage total and truncal fat to the NOR group. Adjusting for sex and/or age did not change these results (data not shown).

Endogenous Glucose Production

EGP_{basal} was significantly higher in the SA than the NOR group, as shown in Table 4 and Figure 1A. This difference remained significant after adjustment for possible confounders, including sex, age, height, weight, BMI, %TBF, FFM, HbA_{1c}, fasting C-peptide, or fasting plasma glucose (data not shown). During clamp hyperinsulinemia the EGP was reduced, the ethnic difference in endogenous glucose production (EGP_{clamp}) was attenuated, and no longer significant.

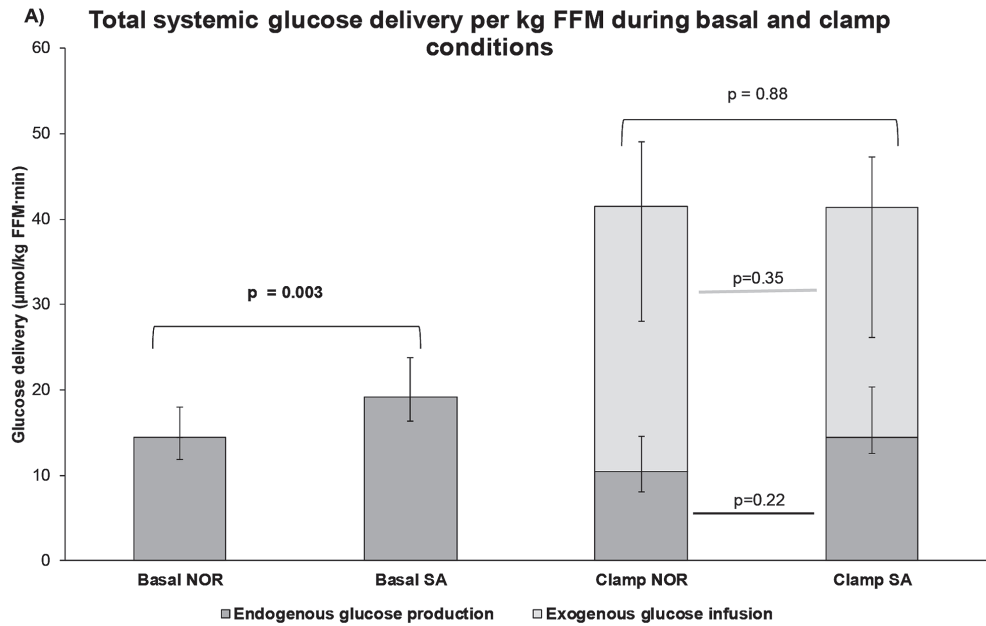
EGP_{clamp} was detectable in all patients, ranging from 3.4% to 90.6% of the total glucose disposal rate (TGD), with a median of 25.8%. In an effort to find predictors of EGP_{clamp} variation, we performed simple correlations between EGP_{clamp} and parameters which could influence $EGP_{clamp.}$ The following parameters correlated to EGP_{clamp} with a *p-value* <0.1: diabetes duration, fasting plasma glucose (FPG), se-insulin at end of clamp, fasting se-C-peptide and HbA_{1c} (Table 5). We then performed an all subsets multiple regression analysis, using $logEGP_{clamp}$ as dependent variable. Log FPG was the only significant parameter to remain, with an unstandardized beta = 0.78, $p = 0.003$ and an R^2 of only 0.15. The separate correlation coefficients in the ethnic subgroups showed differences: FPG and most of the other parameters correlated significantly to EGP_{clamp} only in the NOR group. The correlation between GIR and EGP_{clamp} was neither significant in the total patient group nor in the two separate ethnic subgroups. The correlation between TGD and EGP_{clamp} was significant in the SA subgroup but not the NOR subgroup.

Table 4. Endogenous glucose production, insulin sensitivity and insulin secretion.

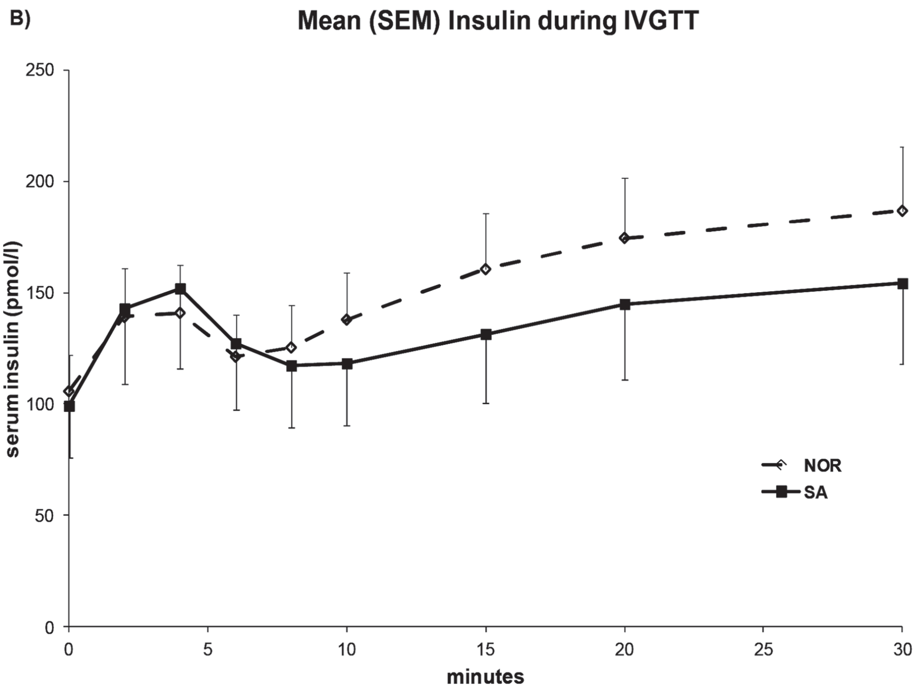
		NOR n = 41	SA n = 18	*p*
EGP_{basal}	μmol/kg FFM·min	14.4 [6.8]	19.1 [9.1]	***0.003***
EGP_{clamp}	μmol/kg FFM·min	8.9 [6.7]	10.8 [10.4]	*0.216*
EGP_{clamp} %	% of TGD	24.9 [24.3]	38.7 [27.7]	*0.107*
GIR	μmol/kg FFM·min	28.9±15.8	24.7±14.6	*0.343*
TGD	μmol/kg FFM·min	39.2±17.6	39.1±20.4	*0.990*
$Se\text{-}insulin_{end\ clamp}$	pM	1290 [425]	1270 [1087]	*0.889*
$AIRg_{0-8\ min}$	$AUC_{0-8\ min}$	124 [275]	220 [302]	*0.352*
$AIRg_{0-30\ min}$	$AUC_{0-30\ min}$	1003 [1505]	852 [1452]	*0.383*
$LogAIRg_{0-8\ min}$	$LogAUC_{0-8\ min}$	2.15±0.52	2.34±0.44	*0.201*
$LogAIRg_{0-30\ min}$	$LogAUC_{0-30\ min}$	3.06±0.36	2.98±0.32	*0.425*

Data are presented as mean ± standard deviation or median [inter-quartile range]. *p*-values from Student's *t*-tests or Mann-Whitney U tests as appropriate. NOR: Nordic, SA: South Asians, EGP: endogenous glucose production, FFM: fat free mass, TGD: total glucose disposal, GIR: glucose infusion rate, AIRg: acute insulin response to glucose, AUC: area under the curve (from 0–8 minutes and 0–30 minutes of the intra-venous glucose tolerance test). For $LogAIRg_{0-8\ min}$ n = 36 NOR and 16 SA, and for $LogAIRg_{0-30\ min}$ n = 40 NOR and 17 SA.

doi:10.1371/journal.pone.0083983.t004



A)
Total systemic glucose delivery per kg FFM during basal and clamp conditions
Glucose delivery (µmol/kg FFM·min)
60
50
40
30
20
10
0
p = 0.003
p = 0.88
p=0.35
p=0.22
Basal NOR
Basal SA
Clamp NOR
Clamp SA
Endogenous glucose production
Exogenous glucose infusion



B)
Mean (SEM) Insulin during IVGTT
serum insulin (pmol/l)
250
200
150
100
50
0
0
5
10
15
20
25
30
minutes
NOR
SA

Figure 1. Glucose delivery and insulin secretion during basal and clamp conditions. A) Median [inter-quartile range] values of basal and clamp glucose delivery per kg fat free mass (FFM), both from endogenous glucose production and exogenous glucose infusion. *p*-values from Mann-Whitney U tests. B) Mean (standard error of mean) serum insulin levels during the 30-minute intra-venous glucose tolerance test. NOR = Nordic, SA = South Asians.
doi:10.1371/journal.pone.0083983.g001

Insulin Sensitivity

There was no significant ethnic difference in insulin sensitivity expressed as the TGD in μmol/kgFFM·min (Table 4, Figure 1A). After adjusting TGD for log EGP_{clamp} (beta = 28.4, *p = 0.001*) and log waist circumference (beta = −114.7, *p = 0.028),* in a multiple regression analysis, ethnicity came closer to significance (beta = −9.1, *p = 0.111).* Further adjusting for age *(p = 0.84)* or sex *(p = 0.97)* was not significant.

Insulin Secretion

All but seven of the 60 subjects where an IVGTT was performed had some preserved first phase insulin secretion, (increased incremental AUC_{0-8}), and two thirds of the patients displayed an $AUC_{0-8}>100$ pM. Insulin secretion (AIRg) did not differ significantly between the two ethnicities (Table 4, Figure 1B). After adjusting for HbA_{1c} in a multiple regression analysis to account for possible glucose toxicity, there was a non-significant trend towards higher insulin secretion in the SA group (beta = 0.30, *p = 0.052*, model significance: *p = 0.030).* $LogAUC_{0-8}$ was the dependent variable and ethnicity and log HbA_{1c} (beta = −2.27, *p = 0.02*) were independent variables. Further adjusting for age *(p = 0.39)* and sex *(p = 0.51)* was not statistically significant. When measured as the AUC_{0-30}, insulin secretion did not differ between the two ethnic groups, neither before nor after adjustment for HbA_{1c}, age and/or sex. A longitudinal analysis of AUC for insulin during the total 30 minutes of IVGTT did not show any significant ethnic difference either (Figure 1B).

Table 5. Correlations to endogenous glucose production during clamp.

		Total patients	NOR	SA
		n = 57	**n = 39**	**n = 18**
Diabetes duration	r_s	0.251	**0.343**	−0.058
	p	*0.059*	***0.033***	*0.819*
Fasting plasma glucose	r_s	**0.420**	**0.524**	0.057
	p	***0.001***	***0.001***	*0.823*
Serum insulin at end of clamp	r_s	**−0.295**	−0.187	−0.387
	p	***0.026***	*0.254*	*0.113*
Fasting serum C-peptide	r_s	**−0.272**	**−0.320**	−0.034
	p	***0.040***	***0.047***	*0.926*
HbA_{1c}	r_s	**0.335**	**0.338**	0.101
	p	***0.011***	***0.035***	*0.689*
Exogenous glucose infusion rate	r_s	−0.035	−0.116	0.228
	p	*0.798*	*0.480*	*0.363*
Total glucose disposal	r_s	**0.318**	0.156	**0.591**
	p	***0.016***	*0.342*	***0.010***

Data are presented as Spearman's correlation coefficients (r_s) with corresponding *p*-values. Significant correlations in bold. NOR: Nordic, SA: South Asians.
doi:10.1371/journal.pone.0083983.t005

Glucose and Fat Oxidation and Non-oxidative Glucose Metabolism

Figure 2 displays glucose and fat metabolism in peripheral tissues in the basal fasting and the hyperinsulinemic clamp state, measured by indirect calorimetry. Figure 2A demonstrates that higher endogenous glucose production in SA leads to increases in both oxidative and non-oxidative metabolism in peripheral tissues. This figure also demonstrates the higher non-oxidative than oxidative metabolism in the basal state in both ethnic groups, and that non-oxidative glucose metabolism increases more than oxidative in the clamp hyperinsulinemic state in both ethnicities.

Basal fat oxidation measured per kg fat free body mass was similar in the two ethnic groups (Table 6, Figure 2B). Fat oxidation decreased during clamp hyperinsulinemia, as glucose metabolism increased. These changes were similar in the two groups.

Basal and Clamp Energy Expenditure

The mean, unadjusted resting energy expenditure (REE) in kJ/day, estimated by indirect calorimetry, was higher in the NOR than in the SA patients (Table 6). However, after adjustment for FFM, FM, age and sex in a one-way ANCOVA analysis, the ethnic difference was attenuated, and no longer significant (*p = 0.51*), with adjusted mean (SEM) values of 7155 (121) kJ/day in NOR and 6954 (239) kJ/day in SA patients.

REE correlated highly with basal fat oxidation ($r_s = 0.48$, *p = 0.002* in NOR and 0.64, *p = 0.014* in SA patients), but not with basal glucose oxidation ($r_s = -0.06$, *p = 0.73*, and −0.10, *p = 0.75*, respectively), or non-oxidative glucose metabolism ($r_s = -0.16$, *p = 0.36*, and $r_s = 0.40$, *p = 0.16* respectively), although SAs showed a stronger correlation between REE and non-oxidative glucose metabolism than the NOR group.

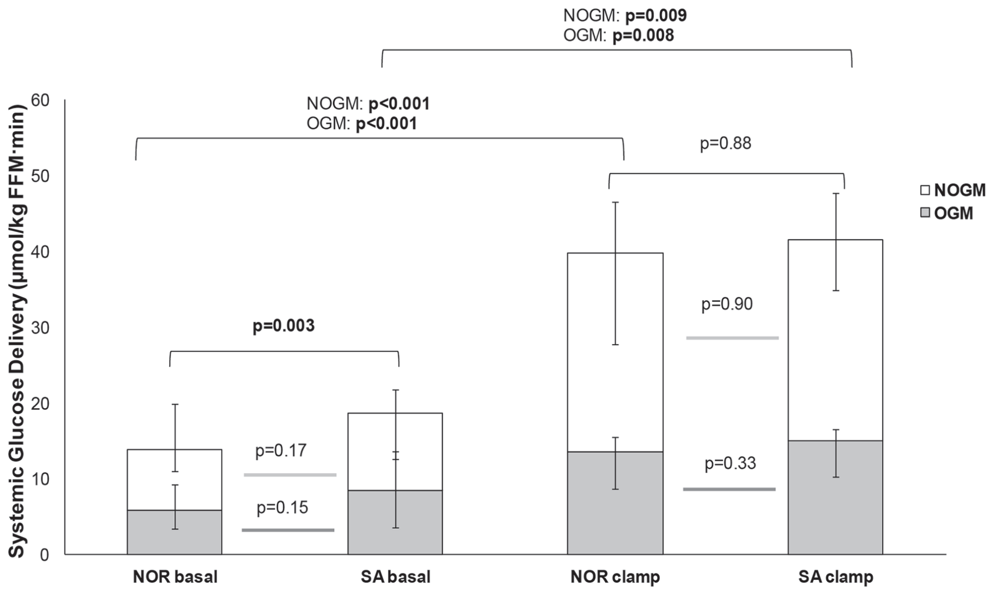
The positive correlation between REE and EGP_{basal} tended to be stronger in SA ($r_s = 0.53$, *p = 0.051*), compared to the NOR subjects ($r_s = -0.18$, *p = 0.28*). The correlation between EE_{clamp} and EGP_{clamp} was also stronger in SA ($r_s = 0.50$, *p = 0.082*), than in NOR subjects ($r_s = -0.06$, *p = 0.74*). Energy expenditure increased significantly during clamp (EE_{clamp}) in the NOR patients (*p = 0.003*), but not in the SA patients (*p = 0.28*). The respiratory quotient (RQ) increased significantly from basal to clamp value in both NOR (*p<0.001*) and SA subjects (*p = 0.008*) (Table 6). This change (ΔRQ) was similar in the two groups.

Relation between Insulin Sensitivity, Insulin Secretion and Vitamin D

Median serum 25-hydroxyvitamin D in the SA group was significantly lower than in the NOR group (table 3). We found no significant correlations between 25-hydroxyvitamin D levels and insulin sensitivity or insulin secretion, neither in the two ethnic groups examined separately, nor in the total cohort.

Discussion

In this study we examined ethnic differences in glucose and fat metabolism and energy expenditure in basal and clamp hyperinsulinemic conditions in subjects with T2D, of SA or NOR ethnicity, living in Oslo, Norway. We found evidence of ethnic differences in fasting endogenous glucose production, and


A)
Oxidative and non-oxidative glucose metabolism
NOGM: p=0.009
OGM: p=0.008
NOGM: p<0.001
OGM: p<0.001
p=0.88
p=0.003
p=0.17
p=0.15
p=0.90
p=0.33
NOGM
OGM
Systemic Glucose Delivery (µmol/kg FFM·min)
0
10
20
30
40
50
60
NOR basal
SA basal
NOR clamp
SA clamp

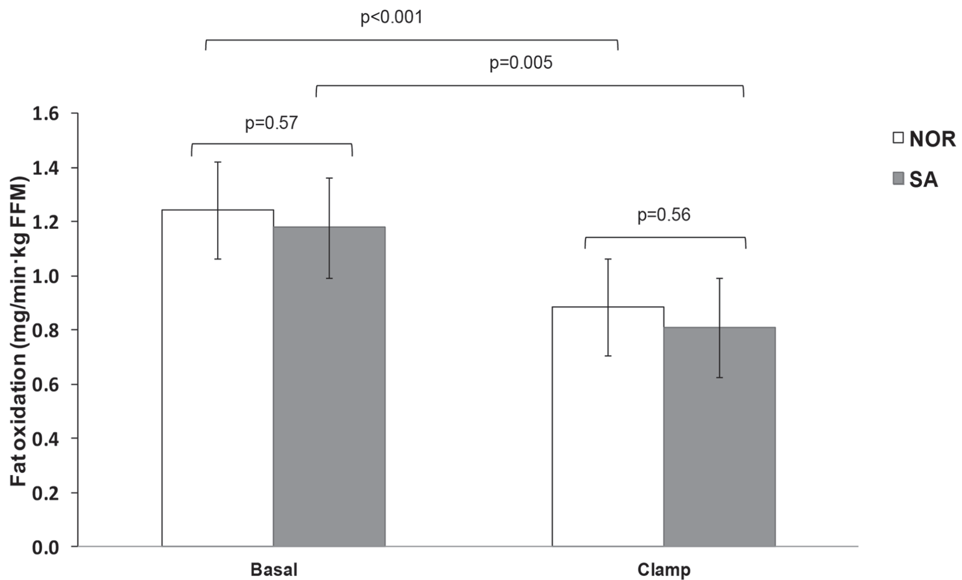

B)
Mean (SEM) fat oxidation in Nordic and South Asian patients
p<0.001
p=0.005
p=0.57
p=0.56
NOR
SA
Fat oxidation (mg/min·kg FFM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Basal
Clamp

Figure 2. Glucose and fat metabolism in Nordic and South Asian subjects with type 2 diabetes. A) Glucose metabolism per kg fat free mass (FFM). Median [inter-quartile range] values of basal and clamp glucose delivery, both from non-oxidative glucose metabolism (NOGM) and oxidative glucose metabolism (OGM) B) Fat oxidation per kg fat free mass (FFM). Mean (standard error of mean) values in basal and clamp conditions. NOR = Nordic, SA = South Asians. Comparisons beween ethnic groups are Student's *t*-tests or Mann-Whitney U tests as appropriate. Comparisons between basal and clamp values are paired samples *t*-tests, after log-transformation where appropriate.
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indications of possible differences in the choice of substrates for energy expenditure both in basal and clamp conditions.

The concept of ethnicity and ethnic groups is complex, consisting of both socio-cultural and biological components that are not clearly defined [12]. The term South Asian ethnicity is often used, although the South Asian region is diverse, with several countries (Pakistan, India, Bangladesh and Sri Lanka), and differences in culture, religion and diet. In diabetes research, using the term South Asian can nonetheless be justified, in view of the fact that the high prevalence of diabetes and increased insulin resistance is present in the whole region [13], particularly in urban areas, and also after migration to Western countries [1].

This study shows a significantly higher fasting EGP in SA compared to NOR patients, which was not explained by any of the examined possible confounding factors. During clamp hyperinsulinemia the EGP was lowered, and the ethnic difference was attenuated. Even so, EGP_{clamp} was still not negligible, and constituted almost 40% of TGD in SA and 25% in NOR patients. Hyperinsulinemia during clamp is often said to suppress EGP almost entirely [14,15], and euglycemic clamp studies are still frequently performed without the measurement of endogenous glucose production [16,17]. However, several authors have demonstrated that EGP_{clamp} persists [18–20]. We here present further evidence that EGP_{clamp} can be substantial in type 2 diabetic patients, even with serum insulin concentrations during clamp as high as 1000–1500 pM. This finding underscores the importance of controlling for hepatic glucose production during clamp studies.

Measuring EGP via the isotope tracer dilution method is both time consuming and costly. In an attempt to find predictors for the estimation of EGP_{clamp} from variables that are easier to measure, we looked at a group of variables which correlated with EGP_{clamp}. Only FPG remained significantly related to EGP_{clamp} in regression analyses, and it explained only 15% of EGP_{clamp} variation in the whole patient group. When looking at the correlations in the separate ethnic subgroups, most variables only correlated significantly in the NOR group. The exogenous glucose infusion rate did not correlate to EGP_{clamp} at all. In the SA group, the only significant correlation was between EGP_{clamp} and TGD, merely reflecting the high percentage of EGP_{clamp} in TGD in this group. We therefore suggest that measuring EGP_{clamp}, in addition to the exogenous glucose infusion rate, is essential for correct estimation of total glucose disposal rate.

Some ethnic groups residing in tropical climates, including SA, have previously been shown to have lower REE than Westerners [21], however, several authors have advocated the need for adjusting REE for fat free mass (FFM) and fat mass (FM), as well as age and sex [4,22]. The lower REE in SA is in this way shown to be due to differences in body composition and not due to ethnicity per se. We find it to be the case also in our study. Our two ethnic groups display clear differences in body composition, and adjusting for these differences, mainly FFM, attenuates the ethnic difference seen in REE. This, however brings us back to the complex concept and definition of ethnicity, whence it can also be argued that lower FFM is an ethnic characteristic of South Asians. This has been described in other studies [23,24].

In basal, resting conditions, energy production is for the most part derived from lipids, and less from carbohydrates [25]. This is also reflected in our study, by the highly significant correlation between REE and fat oxidation in both ethnicities. In the SA subgroup, however, there is also a near-significant correlation between REE and EGP_{basal}. This could signify increased use of carbohydrates as energy substrate in the fasting state in SA, to such an extent that it becomes important for the total REE. However, we did not find any ethnic differences in basal RQ or in ΔRQ from fasting to clamp hyperinsulinemic conditions, that would have clearly indicated an ethnic

Table 6. Basal and Clamp Indirect Calorimetry.

		NOR n = 38	SA n = 14	*p*
Basal glucose oxidation	µmol/kg FFM·min	6.5±4.5	8.5±4.0	*0.151*
Basal non-oxidative glucose consumption	µmol/kg FFM·min	9.0±5.9	12.0±9.1	*0.167*
Basal fat oxidation	mg/kgFFM·min	1.24±0.37	1.18±0.36	*0.572*
Clamp glucose oxidation	µmol/kg FFM·min	12.7±4.6	14.2±6.7	*0.331*
Clamp non-oxidative glucose consumption	µmol/kg FFM·min	26.3 [17.6]	26.6 [29.5]	*0.897*
Clamp fat oxidation	mg/kgFFM·min	0.89±0.38	0.81±0.50	*0.557*
REE	kJ/day	7465±1202	6104±1214	***0.001***
EE_{clamp}	kJ/day	7750±1315	6263±1139	***<0.001***
RQ_{basal}		0.79±0.05	0.80±0.04	*0.201*
RQ_{clamp}		0.84±0.05	0.86±0.06	*0.440*
ΔRQ		0.052±0.035	0.053±0.062	*0.994*

Data are presented as mean ± standard deviation or median [inter-quartile range]. *p*-values from Student's *t*-tests or Mann-Whitney U tests as appropriate. NOR: Nordic, SA: South Asians, FFM: fat free mass, REE: resting energy expenditure, EE_{clamp}: energy expenditure during clamp, RQ: respiratory quotient, ΔRQ: change in respiratory quotient from basal to clamp conditions.
doi:10.1371/journal.pone.0083983.t006

difference in metabolic flexibility. The values of basal RQ and ΔRQ in our subjects were comparable to the group with diabetes in the recently published study by van de Weijer [26]. In that study, they also demonstrated that insulin stimulated RQ is mainly dependent on glucose disposal rates, which in our study are similar in the two groups.

Our NOR participants are significantly more obese than the SA, which could in part explain why there is no obvious ethnic difference in TGD. After adjustment for difference in waist circumference, as well as the endogenous glucose production, the ethnic difference in TGD came closer to significance.

In our study we found a non-significant trend towards higher both oxidative and non-oxidative glucose metabolism in SA compared to NOR subjects in the basal, post-absorptive state. The total EGP_{basal} was significantly higher. This points towards a higher degree of hepatic insulin resistance in SA. One could speculate that a possible higher basal oxidative glucose metabolism in muscle, triggered by increased substrate availability from fasting hyperglycemia, leads to less use of lipids as energy substrate, again leading to lipid accumulation and further aggravation of the hepatic insulin resistance, as described in the metabolic inflexibility hypothesis [3]. In post-absorptive conditions skeletal muscle contribution to total metabolism is modest [3]. A possible ethnic difference in muscle metabolism could thus have been masked.

The hepatic insulin resistance could also initially have been caused by increased lipid storage in the liver due to adipose tissue overflow [2]. Percentage truncal fat was similar in our two ethnic groups, although the NOR group was significantly more obese. In a previous study we found that even though a group of Pakistani and Norwegian subjects with T2D had similar abdominal adipose tissue distribution, the visceral adipose tissue was more metabolically active in the Pakistani subjects (Wium C, Eggesbo HB, Ueland T et al, 2013, unpublished data). An increased supply of NEFA from visceral adipose tissue to the liver would, in addition to the effect of increasing the insulin resistance, constitute a source of substrate for gluconeogenesis.

Gluconeogenesis is known to be increased in subjects with T2D, being in large part responsible for the increased post-absorptive EGP. When using [6,6-$^2H_2$] glucose as tracer, Cori cycling is included in the estimation of the total glucose disposal, and has been shown to be increased 25% in T2D in general [27]. It is possible that the increase in non-oxidative glucose metabolism in SA in large part corresponds to increased Cori cycling, due to substrate availability through hyperglycemia in tissues, with lactic acid production in muscle or other tissues by anaerobic glycolysis, then transport back to the liver and re-use as substrate in gluconeogenesis, creating a vicious circle.

The data in this study are baseline results from a vitamin D intervention trial. It was therefore of interest to look for associations between baseline 25-hydroxyvitamin D levels and measures of insulin sensitivity and insulin secretion. We found significantly lower median 25-hydroxyvitamin D levels in the SA group. Could differences in vitamin D status explain some of the ethnic differences in glucose metabolism? Several epidemiological studies have in recent years shown a relationship between vitamin D and diabetes [28,29], the metabolic syndrome [28], insulin resistance [30,31], and some studies also with insulin secretion [32]. However, in most of the published studies that report significant associations, or an effect of vitamin D intervention, the primary end points have been surrogate markers based on fasting blood values, like the HOMA indices [28,33,34]. The few studies using more sophisticated methods, like OGTT, IVGTT or clamps have usually not been able to show similar significant relationships [35–39]. We did not find any association between levels of 25-hydroxyvitamin D and TGD or AIRg. The question therefore still remains whether there is a genuine and causal relationship between vitamin D and diabetes, and we have to await results from randomised, controlled trials.

A strength of this study was the use of gold standard methods such as the euglycemic clamp with tracer dilution, coupled with indirect calorimetry. This enabled us to measure both fasting endogenous glucose production and insulin sensitivity, as well as carbohydrate and fat metabolism, both in the basal and hyperinsulinemic state. To our knowledge, this has not been reported in SA subjects with T2D previously. The patients were included by a large variety of approaches, with wide inclusion criteria, the main restriction being the 25-hydroxyvitamin D levels $\leq$50 nM, with the aim of securing broad representativeness. The following limitations must be noted: The data presented here are cross-sectional. No efforts towards matching of the two ethnic groups at baseline were made. The inclusion of SA patients in the study proved challenging, hence the SA group was limited in size, increasing the risk of Type II statistical errors. Further studies in a larger group of patients, will therefore be necessary to confirm some of the findings that are still uncertain in our study. We selected only subjects with low levels of vitamin D, and our results therefore cannot be generalized to the whole population of subjects with T2D, although low vitamin D levels are common i T2D. Nevertheless, we did not find any correlation between 25-hydroxyvitamin D levels and insulin sensitivity or insulin secretion. This is an exploratory study, and we have judged it appropriate not to show p-values corrected for multiple testing. Bonferroni-Holm corrections were performed, showing that p-values <0.01 remained significant. However, due to the high risk of missing a genuine difference that is clinically significant, we still focus on the non-corrected tests [40]. Hence, there is also a risk of reporting p-values <0.05 by chance.

Conclusions

We have demonstrated higher basal EGP in SA patients with established T2D. Clamp EGP can be substantial in patients with established T2D, and cannot be estimated from the surrogate markers measured. We found no ethnic difference in insulin sensitivity or in first phase insulin secretion. Findings of near significant correlations between REE, EE_{clamp} and EGP in the SA group only, might indicate increased post-absorptive glucose metabolism in the SA group, at the expense of lipid metabolism, but these results have to be confirmed in larger studies. Finally, we found no indication of any relation between vitamin D and insulin sensitivity and secretion.

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Author Contributions

Conceived and designed the experiments: CW HLG EFE KIB. Performed the experiments: CW HLG. Analyzed the data: CW HLG KIB. Wrote the paper: CW HLG. Critical revision of manuscript: CW HLG EFE KIB. Final approval of manuscript: CW HLG EFE KIB.

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