#### **Diabetes & Vascular Disease Research**

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### Memories of Gerald Reaven (28th July 1932-12th February 2018)

Personal	Memories

### **Gerald Reaven: A man for our times**

#### Yehuda Handelsman



Professor Gerald M Reaven 28 July 1928–12 February 2018.

Professor in Medicine, Stanford University, School of Medicine, Stanford, CA, USA.

2018 was a distinct year; on 12 February, Gerald M Reaven – Jerry – passed away, peacefully at home. The year was marked with anniversaries. It was the 30-year anniversary to Gerald Reaven's Banting lecture 1988: Role of Insulin Resistance in Human Disease where he introduced 'Syndrome X', and when I saw Jerry for the first time – an occasion I'll discuss later. It was the 20-year anniversary to our partnership in education and scientific initiatives. 2018 also marked the 15-year anniversary of the founding of The World Congress on Insulin Resistance Diabetes and Cardiovascular Disease (WCIRDC).

The 16th WCIRDC, 29 November–1 December 2018, was dedicated to honour and celebrate Jerry's science and life. There was a special session 'The Metabolic Syndrome Revisited: A Salute to Gerald Reaven, MD' chaired by Ralph DeFronzo and Peter Grant and included a presentation by Peter Reaven, an endocrinologist – Jerry's son, as well as lectures by Jerry's colleges, corroborators and past fellows and the renamed award and keynote lecture: 'Gerald Reaven Distinguished Leader in Insulin Resistance'.

#### The beginning

Jerry was born in Gary, IN, USA, grew up in Cleveland, OH, USA, where he developed his love for baseball. He attended the University of Chicago as an undergraduate and for medical school. After serving in the US Army Medical Corps in Europe, he completed his internal Diabetes & Vascular Disease Research 2019, Vol. 16(2) 113–115 © The Author(s) 2019 Article reuse guidelines: sagepub.com/journals-permissions DOI: 10.1177/1479164119827769 journals.sagepub.com/home/dvr

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medicine residency at the University of Michigan in Ann Arbor. He joined the Stanford School of Medicine faculty in 1960, in the endocrinology division. He progressed to a full professorship in 1970. Jerry led endocrinology and gerontology eventually, after semi-retirement, he joined the cardiovascular division. Jerry was captivated by the findings of Himsworth, in the mid 1930s, that a large number of patients with diabetes are 'insulin insensitive',1 a finding that went dormant for many years. In 1970, Reaven argued for the existence of insulin resistance (IR), a diminished response to the hormone insulin, in people with type 2 diabetes mellitus (DM). At that time, a controversial concept was met with huge opposition. Jerry demonstrated, by a quantitative method to measure insulin-mediated glucose uptake in humans, that there are variable degrees of IR in healthy population and those with type 2 diabetes (T2D). In 1984, the National Diabetes Data Group endorsed the concept and referred to T2D as non-insulin-dependent diabetes mellitus (NIDDM) and that resistance to insulinstimulated glucose uptake is a characteristic finding in patients with NIDDM and impaired glucose tolerance. Jerry did not stop there; he went further to show how IR and hyperinsulinaemia have a role in cardiovascular disease (CVD) in people who do not have diabetes.

#### When we met

I recently re-read the 1988 publication of Jerry's Banting Lecture 1988: Role of Insulin Resistance in Human Disease.<sup>2</sup> I was amazed at his vision and foresight. In today's medical literature, where 3 years is already old news, this 30-year-old paper represents science which is as fresh and relevant today. In his lecture, Jerry introduced the novel idea of a link between IR and a cluster of metabolic abnormalities that together greatly increased the risk for CVD. He concluded that there is a series of related variables – which he named syndrome X – that tends to occur in the same individual and may be important in the aetiology of coronary artery disease (CAD). These changes

The Metabolic Institute of America, Tarzana, CA. USA

Corresponding author:

Yehuda Handelsman, The Metabolic Institute of America, 18372 Clark St #212, Tarzana, CA 91356, USA. Email: yhandelsman@gmail.com

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included hyperglycaemia, hyperinsulinaemia (the corollary of IR), an increased triglyceride (TGL), a decreased high-density lipoprotein cholesterol (HDL-C) and high blood pressure. The common feature of the proposed syndrome is IR, and all other changes are secondary to it. All five of the proposed consequences have been shown to increase the risk of CAD.

I met Jerry at the Medical School of the University of Southern California where I was a first-year fellow in a unique diabetes-endocrinology programme. At our weekly endocrine fellows' grand round, Jerry, following his 1988 Banting Award, lectured on the relationship of insulin to HTN. Like so many others, those days we just did not get it, we laughed. I actually thought that it was some kind of a hoax that he was just using his stature to lecture on issues regardless of how scientifically sound they were.

And yet, his lecture stuck with me. Couple of years later, when I was already in clinical practice, I started reading Jerry's and others' work on IR and suddenly everything clicked. I became a fan. In fact, I utilized the IR concepts - though primarily researched based, in my practice. As such, I was an early adopter of Metformin (introduced in the United States only in 1995) and thiazolidinediones (TZDs). Couple of years later, Jerry and I met at a small medical meeting in Palm Springs. Jerry was a speaker, and he came with his wife Eve. I was with my wife and our two small kids. Jerry had a name as a tyrant, a very hard person to work with, not pleasant to fellows, faculty and colleagues. People were fearful of him. I was not aware of any of it. I found his lecture to be great. At the end of the day, there was a group dinner. A whole ballroom for a mere 30 people or less. We were first with the kids; suddenly a woman comes down, she looked around, all the tables were empty and asked if she could join us. 'Of course'. 'I hope you won't change your mind when my husband joins us'surely Eve was aware of Jerry's reputation. When Jerry came, he and I went for a drink, turns out we both liked gin martini with olives - a good way to start a relationship with Jerry. Over drinks, I told him of what I had thought of him, and his work, when I heard him first. He laughed, and we became friends.

#### **Developing education and initiatives**

We shared similar views on the role of IR, Jerry as a researcher and I as a clinician. We named our first education meeting in 1998 'Syndrome X, Diabetes and beyond' with a focus on CVD. In the following years, we kept searching our goals based on published data and 'popular' directions. In 1999, we called the meeting 'Syndrome X, Diabetes, Obesity & The Heart'; in 2000, we added HTN to the name. In 2001, we changed to 'The Metabolic- Insulin Resistance- Syndrome X'. At that time, the NCEP/ATPIII published their definition of the metabolic syndrome. In fact, following Jerry's 1988 syndrome X, the World health Organization (WHO) published in 1998 their version of the syndrome which they already called metabolic syndrome, followed by the EGIR: European Group for Insulin Resistance that also used the name metabolic syndrome with similar definition. The AACE/ACE (American Association of Clinical Endocrinologists/American College of Endocrinology) convened a consensus to address syndrome X versus metabolic syndrome. I recruited Jerry to join ACCE and be a co-chair with Dan Einhorn and myself. The AACE/ACE elected to call it the insulin resistance syndrome (IRS) over the metabolic syndrome.<sup>3</sup> Although we recognized the clinical utility of metabolic syndrome, with criteria similar to the Metabolic Syndrome, to identify people at risk for Diabetes and CVD, we preferred the IRS not just because it presents the pathophysiologic role of IR, but, more importantly, it extends its clinical impact to many other conditions beyond DM and CVD.

#### The IRS

IR and hyperinsulinaemia presentations is often determined by the difference in resistance of various tissues and organs. Many, if not most, of the adverse events attributed to IR are secondary to the effects of compensatory hyperinsulinaemia (an attempt at preventing the decompensation of glucose homeostasis) on tissues that still have normal insulin sensitivity. Compensatory hyperinsulinaemia acts on the kidney to retain salt and water, which may explain the development of essential hypertension, and it decreases uric acid clearance by the kidneys, increases sympathetic nervous system activity and increases prevalence of certain cancers. It also impacts the ovaries where insulin stimulates testosterone secretion, as well as affecting other glands including the thyroid. Following Jerry's research, he proved Himsworth's concept that IR is part of the pathophysiology of T2DM. Jerry than extended the concept to syndrome X - linking IR to CVD, eventually demonstrating that the IRS leads to many other associated conditions: hypertension, coronary artery disease, polycystic ovarian syndrome (PCOS), non-alcoholic liver disease (NAFLD), certain forms of cancer and obstructive sleep apnoea, congestive heart failure and cognition.<sup>4</sup>

Jerry was often misunderstood. Although he recognized the impact of obesity on IR, he also differentiated obesity from IR, specifically highlighting the risk of IR in normalweight patients. He recognized the importance of the metabolic syndrome although he preferred the more inclusive IRS. He, and I and AACE included hyperglycaemia, but not diabetes as part of the syndrome. We believed that once diabetes develops, we have approved medications and guidelines to manage the condition, while there are currently no approved medications for the IRS. Our concern was to be able to identify them early, intervene and prevent the development of DM and CVD. However, because of Jerry's combative – debate like – preaching of his

#### Handelsman

concepts, he was wrongly accused for discounting obesity and metabolic syndrome. The criticism did not sway him, and he has always fought for his – pure – science and databased principles.<sup>5</sup>

# The world congress of IR diabetes and CVD

In 2003, we agreed that we should elevate our education efforts. There was a great need to understand the many faces of IR. To both myself, a community physician, and Jerry, from academia, there was a need to extend the impact of IR from the research arena to clinical practice. To be able to cover all the fields which relate to IR, we founded the International Committee for Insulin Resistance (ICIR) with experts in diabetes, cardiology, obesity, lipids, cancer, PCOS, liver, paediatrics as well as researchers and clinicians. At Jerry's suggestion, we started an abstract programme, initially with the journal Diabetes and Vascular Disease Research, followed by Endocrine Practice. Jerry provided vision and leadership, teaching me the unwavering search for truth, to be scientifically honest and follow the data. The World Congress on Insulin Resistance Syndrome DM & CVD has become the main stage where globally recognized scientists and clinicians present and share their knowledge. As the congress progressed, its strength became from its fabulous international faculty. A lot of this success was due to following Jerry's principle 'if the speaker does not have anything of value to say- why should he or she say it here?' Scientifically, the congress developed from a mere 'Getting to the Heart of the Matter' to 'Exploring New Frontiers in Metabolism-Tomorrow's Clinical Science Today'.

#### Friendship

Jerry proved to be a renaissance man, knowledgeable, witty and with an exquisite taste in food, drinks, music and life. He had the reputation of being rough and unkind, and though he could be difficult and demanding at times, I actually found him to be warm, generous, social and helpful. His intelligence and analytical mind contributed greatly to the many conversations we had. We typically met several times a year. Two of these yearly meetings became a tradition. I would fly to San Francisco and Jerry would pick me up at the airport. His car was the only car one could buy which was all 'no power': stick shift, no automatic windows, no power breaks nor steering, a regular lock and obviously no navigation. Generally, he left his cell phone at home. We would drive around till we found a decent looking fast food place. We would sit for about 5h on coffee and food. Jerry had an incredible knowledge of the literature while I updated him on leadership changes within medical societies and academia. At the end of the meeting, we would create the framework for the next congress. The second meeting was the evening before the World Congress. We met over a couple of gin martinis updating each other in a more relaxing social atmosphere getting ready for the conference. Jerry was most happy when Eve joined him. Eve was a scientist with specialty in electronic microscopy, who turned an artist. She created beautiful ties and scarfs from photos she took with the electronic microscope. Jerry often negotiated for her to get a booth in large meetings, where he was lecturing, allowing her to sell her art. At times, he would replace her and himself sell the ties and scarfs. My wife Nava is a painter artist herself, and we added another dimension to the congress, an art and science exhibition displaying both Nava and Eve's work.

Jerry Reaven is one of America's Scientific Giants, I was fortunate to be associated with him for 20 years, learn from him, adopt him as my mentor and had the privilege to call him my friend or in Jerry's language 'buddy'.

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# The Reaven syndrome: An historical perspective

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#### Handrean Soran<sup>1,2</sup>, Safwaan Adam<sup>1,2</sup>, Jan H Ho<sup>1,2</sup> and Paul N Durrington<sup>1</sup>

When reliable plasma insulin assays and accurate triglyceride methods became available in the 1960s, the scene was set for Gerald Reaven and colleagues to discover the association between the insulin response to carbohydrate feeding and serum triglyceride levels.1 Higher insulin responses were associated with higher triglyceride levels. Initially, Reaven hypothesised that the increased insulin levels were the cause of the hypertriglyceridaemia, because insulin was believed at that time to stimulate hepatic very low density lipoprotein (VLDL) secretion.<sup>1</sup> However, in the 1980s, it became possible to culture adult hepatocytes without the necessity for insulin to maintain their viability and it was then evident that the primary effect of insulin on hepatic VLDL secretion was inhibitory.<sup>2</sup> This proved to be due to an increase in the proteolytic degradation of newly synthesised apolipoprotein B100 [the major protein moiety of VLDL and low-density lipoprotein (LDL)] before it could be assembled into VLDL.3 Thus, hypertriglyceridaemia must be due not to hyperinsulinaemia, but to resistance to insulin. Reaven then argued that, like muscle, the liver must be resistant to the action of insulin, at least in regard to its diminished capacity to take up glucose. Increased insulin levels were thus a response to overcome this resistance in order to maintain euglycaemia [or the failed attempt to maintain normal glucose levels in the case of type 2 diabetes mellitus (T2DM)]. Hepatic insulin resistance releases the brake imposed by insulin on VLDL production and thus explains the hypertriglyceridaemia of metabolic syndrome and T2DM. This theory was later confirmed by human studies of VLDL kinetics.3 Subsequently, a clinical syndrome emerged associated with an exaggerated insulin response to carbohydrate feeding. In addition to hypertriglyceridaemia, this syndrome comprised increased risk of atherosclerotic cardiovascular disease (CVD), T2DM or a predisposition to develop it, low high-density lipoprotein (HDL) cholesterol, non-alcoholic steatohepatitis, hypertension, hyperuricaemia, raised indices of inflammation and of coagulation (plasminogen activator inhibitor-1, fibrinogen), hirsutes and male pattern obesity in women [polycystic ovary syndrome, low sex hormone binding globulin (SHBG)] and in extreme cases acanthosis nigricans.<sup>3,4</sup> This, Reaven termed 'Syndrome X',<sup>2</sup> although it is now more widely known as the metabolic syndrome, particularly when associated with central

obesity. That insulin resistance and the hyperinsulinaemia arising as a consequence are the causes of this syndrome is the current iteration of the Reaven hypothesis. Visceral adipose tissue is believed to release inflammatory cytokines, which, arriving at the liver in high concentration via the portal vein, oppose the anabolic actions of insulin.

Diabetologists will be familiar with the large doses of insulin required to make even modest improvements in hyperglycaemia in obese patients with T2DM, often far greater than are required in type 1 diabetes. However, while Reaven was developing his hypothesis, rarer syndromes involving insulin resistance came to light in which a hundred or more units of exogenous insulin may be required each day. Among these are insulin receptor mutations, which lead to hyperglycaemia, but not hypertriglyceridaemia, and abnormalities of body fat distribution, such as Dunnigan-Kobberling syndrome (due most commonly to mutation of LMNA which codes for lamins of the inner nuclear membrane) in which the insulin resistance is associated with both hyperglycaemia and hypertriglyceridaemia.5 It thus became obvious that resistance to insulin-stimulated glucose uptake (and consequent increased insulin secretion) could arise at the pre-receptor level [e.g. by non-esterified fatty acid inhibition of glucose uptake (Randle effect)], at the level of the insulin receptor (due say to a gene variant) or occur as post-receptor phenomena within the cell where insulin signals to many processes by a variety of mechanisms. Indeed, when the insulin receptor is intact, some of these processes may be underactive due to insulin resistance to their particular signalling mechanism while others may be overstimulated because their regulatory pathway can still respond to the raised insulin levels. Furthermore, these

**Corresponding author:** 

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<sup>&</sup>lt;sup>1</sup>Core Technology Facility, Cardiovascular Research Group, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK

<sup>&</sup>lt;sup>2</sup>Cardiovascular Trials Unit, Manchester University NHS Foundation Trust, Manchester, UK

Paul N Durrington, Core Technology Facility, Cardiovascular Research Group, Faculty of Biology, Medicine and Health, The University of Manchester, 46 Grafton Street, Manchester M13 9WL, UK. Email: pdurrington@manchester.ac.uk

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effects might vary in different tissues. Ideas such as these might provide some resolution of the conflict which exists between which components of the Reaven syndrome are due to resistance to insulin (too little insulin action) and which are due to the consequent hyperinsulinaemia (too much insulin action). For example, SHBG is decreased in insulin resistance, leading to increased free androgen levels in both men and women. This at least partly explains the androgenisation of insulin-resistant women and thus their male pattern (visceral; central) obesity and hirsutes. Despite the association of insulin resistance with decreased SHBG, however, tissue culture experiments with human hepatocytes reveal insulin to have an inhibitory action on SHBG production. Thus, unlike the VLDL production pathway where insulin resistance decreases the inhibitory effect of insulin, the pathway for the production of SHBG must escape resistance to the action of insulin and be inhibited by the hyperinsulinaemia which occurs to overcome resistance to glucose uptake. Also acanthosis nigricans (a cutaneous disorder manifested by symmetric, hypertrophic, papillomatous, velvety, hyperpigmented plaques commonly found in the axillae and on flexural and intertriginous areas) appears secondary to excessive insulin concentrations.

Reaven's hypothesis has generated a substantial body of research and has stood the test of time better than many scientific concepts and continues to provide insights into atherogenic mechanisms and to stimulate many new lines of enquiry. An outstanding, fundamental issue relating to metabolic syndrome is which came first – the insulin resistance or the predisposition to deposit fat centrally rather than peripherally which then leads to insulin resistance? If androgenisation is secondary to insulin resistance, then it cannot explain a predisposition to deposit fat preferentially in the abdomen before insulin resistance has occurred. Recently, genetic antecedents of visceral as opposed to peripheral obesity have been revealed, which are likely to provide a more satisfactory explanation in individuals susceptible to metabolic syndrome.<sup>6</sup>

Traditionally treatment has been directed at individual components of the metabolic syndrome, such as hyperglycaemia, dyslipidaemia or hypertension. The syndrome is not only frequently occurring in people with T2DM and CVD but is present before either are clinically evident. It thus presents an opportunity for disease prevention, certainly of central obesity, its most common cause. Treating this with diet, medication or surgery is thus the most obvious therapeutic approach. New targets for pharmacological intervention are likely, however, to be identified from patients with a substantial genetic component to their insulin resistance. These may lead to the development of novel therapies which could prove more generally applicable to the syndrome first fully recognised by Gerald Reaven.

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### Insulin resistance and insulin hypersecretion in the metabolic syndrome and type 2 diabetes: Time for a conceptual framework shift

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#### Christopher J Nolan<sup>1,2</sup> and Marc Prentki<sup>3,4</sup>

#### Abstract

While few dispute the existence of the metabolic syndrome as a clustering of factors indicative of poor metabolic health, its utility above that of its individual components in the clinical care of individual patients is questioned. This is likely a consequence of the failure of clinicians and scientists to agree on a unifying mechanism to explain the metabolic syndrome. Insulin resistance has most commonly been proposed for this role and is generally considered to be a root causative factor for not only metabolic syndrome but also for its associated conditions of non-alcoholic fatty liver disease (NAFLD), polycystic ovary syndrome (PCOS), obesity-related type 2 diabetes (T2D) and atherosclerotic cardiovascular disease (ASCVD). An alternative view, for which evidence is mounting, is that hyper-responsiveness of islet  $\beta$ -cells to a hostile environment, such as westernised lifestyle, is primary and that the resulting hyperinsulinaemia drives the other components of the metabolic syndrome. Importantly, within this new conceptual framework, insulin resistance, while always a biomarker and state of poor metabolic health, is not considered to be harmful, but a protective adaptive response of critical tissues including the myocardium against insulin-induced metabolic stress. This major shift in how metabolic syndrome can be considered puts insulin hypersecretion into position as the unifying mechanism. If shown to be correct, this new conceptual framework has major implications for the future prevention and management of the metabolic syndrome, including its associated conditions of NAFLD, PCOS, obesity-related T2D and ASCVD.

#### **Keywords**

Cardiovascular diseases, insulin hypersecretion, insulin-mediated metabolic stress, insulin resistance, metabolic syndrome, non-alcoholic fatty liver disease, polycystic ovary syndrome, type 2 diabetes

Professor Gerald 'Jerry' Reaven, in his 1988 American Diabetes Association Banting Lecture titled 'Role of insulin resistance in human disease', showed strong associations between insulin resistance, hyperinsulinaemia, glucose intolerance, hypertriglyceridaemia, reduced highdensity lipoprotein cholesterol and hypertension.<sup>1</sup> He termed the clustering of these factors 'syndrome X' and demonstrated links between this syndrome and increased risk of atherosclerotic cardiovascular disease (ASCVD).<sup>1</sup> Syndrome X, renamed 'metabolic syndrome' (MetS), has been expanded to include additional factors such as central or visceral adiposity, increased apolipoprotein B and small dense low-density lipoprotein (LDL) particles (proatherogenic), elevated plasma fibrinogen and plasminogen activator inhibitor (PAI)-1 (prothrombotic), increased C-reactive protein and inflammatory cytokines (systemic inflammation) and microalbuminuria.<sup>2,3</sup> It is generally accepted that the clustered components of the MetS, including insulin resistance, contribute to the pathogenesis of conditions such as non-alcoholic fatty liver disease (NAFLD), polycystic ovary syndrome (PCOS), type 2 diabetes (T2D) and

Department of Endocrinology, The Canberra Hospital, Garran, ACT, Australia

<sup>2</sup>Australian National University Medical School and John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

<sup>3</sup>CRCHUM and Montreal Diabetes Research Center, University of Montreal, Montreal, QC, Canada

<sup>4</sup>Department of Nutrition and Department of Biochemistry and Molecular Medicine, University of Montreal, Montreal, QC, Canada

#### **Corresponding authors:**

۲

Christopher J Nolan, Department of Endocrinology, The Canberra Hospital, Yamba Drive, Garran, ACT 2606, Australia. Email: christopher.nolan@anu.edu.au

Marc Prentki, CRCHUM and Montreal Diabetes Research Center, University of Montreal, 900 St-Denis, Room R08.412, Montreal, QC H2X 0A9, Canada. Email: marc.prentki@umontreal.ca ASCVD.<sup>2–5</sup> MetS has also been associated with increased risk for chronic kidney disease, cognitive impairment, obstructive sleep apnoea and chronic respiratory diseases.<sup>6–9</sup> While the usefulness of a diagnosis of MetS over its individual components in predicting T2D and ASCVD has been questioned, MetS is now listed as a disease entity (E88.81) in the International Classification of Diseases – 10th Revision (ICD-10), avowing to the importance of Reaven's contribution in bringing this clustering of factors involved in cardiometabolic diseases to the attention of clinicians and scientists.<sup>1,10</sup>

#### Insulin resistance: root cause of MetS and T2D or a protective adaptive response?

Ongoing controversy surrounding the MetS, in terms of its predictive value for particular diseases, is a consequence of the failure of metabolic scientists and clinicians to establish it as a precise condition or to provide a unifying mechanism to explain its clustering of factors, with insulin resistance and visceral adiposity being most commonly proposed.<sup>2,10</sup> Reaven argued for insulin resistance as the unifying mechanism or primary causal factor and, supporting this view, the European Group for the Study of Insulin Resistance proposed 'insulin resistance syndrome' as an alternate name for MetS.<sup>1,11</sup> Furthermore, the mainstream understanding of pathogenesis of T2D is that it develops as a consequence of failure of pancreatic islet  $\beta$ -cells to sustain the hyperinsulinaemia required to compensate for insulin resistance, giving insulin resistance a high-level causative role.<sup>12,13</sup> Thus, within the current conceptual framework, insulin resistance is considered to be 'harmful' and the root cause of T2D and all the other conditions linked to the MetS; furthermore, it should be overcome at any cost.

An alternate view gaining momentum is that insulin resistance has a role in protecting critical tissues of the body from metabolic injury in situations of chronic nutrient excess.<sup>14–17</sup> Its presence within the MetS, while indicative and a biomarker of poor metabolic health, does not mean insulin resistance has a causative role. Furthermore, if insulin resistance does have an adaptive protective role, attempts to override it in patient treatment have the potential to cause harm. Thus, we believe a shift is needed in the conceptual framework by which we understand insulin resistance and the aetiology of T2D and that this has implications on safe management of patients with MetS, T2D and related conditions.

## Insulin sensitivity: adaptable to physiological demands

Physiological adaptability in insulin sensitivity is an important mechanism by which the body can regulate

nutrient partitioning between tissues, necessitated by wide fluctuations in dietary intake and physical activity, and life events such as rapid pubertal growth, pregnancy, illness and ageing. For example, in response to short-term overfeeding, a rapid fall in insulin sensitivity occurs which allows diversion of nutrients from skeletal muscle to adipose tissue for storage, potentially important moving between situations of feast and famine.<sup>18,19</sup> Pregnancy necessitates diversion of nutrients to the developing foetus and insulin resistance in the mother is a mechanism by which this is achieved.<sup>20</sup> Key to this discussion is the role of adaption in insulin sensitivity to a chronic nutrient oversupply, as occurs in westernised lifestyles. As discussed below, the development of insulin resistance in such situations could provide important protection to critical tissues such as the heart from nutrient overload and toxicity.14-17

#### Insulin resistance: a protective mechanism against nutrient-induced intracellular metabolic stress

We previously proposed that in response to chronic over-nutrition, tissues normally responsive to insulin for glucose uptake, such as the heart and skeletal muscle, protect themselves from nutrient-induced toxicity by becoming insulin resistant.<sup>14,15</sup> Without this mechanism at times of nutrient surplus, or by overriding this protective insulin resistance with high-dose insulin therapy, these tissues will be damaged by nutrient overload, a process we have termed 'insulin-induced metabolic stress' (Figure 1).<sup>14,15</sup>

A failure to limit excess entry of glucose at times of concomitant high free fatty acid (FFA) availability will cause cell injury by the mechanisms of glucolipotoxicity.<sup>12</sup> High FFA availability will inhibit glucose oxidation at the level of pyruvate dehydrogenase, such that a high glucose flux will be forced into pathways above this step, including glycogen synthesis, the polyol and hexosamine pathways, and the production of advanced glycation end product (AGE) precursors (Figure 1).<sup>21,22</sup> Similarly, high glucose availability, via malonyl-CoA/AMPK metabolic sensing mechanisms, will inhibit FFA oxidation such that intracellular FFA metabolism will be pushed towards esterification and other processes causing intracellular steatosis and accumulation of complex lipids such as diacylglycerols, cholesterol esters and ceramides (Figure 1).23,24 An excessive mixed nutrient entry into cells will also overload the electron transfer chain resulting in mitochondrial dysfunction and increased reactive oxygen species (ROS) production.<sup>25,26</sup> Endoplasmic reticulum stress and activation of the inflammasome are also known consequences of excessive nutrient entry.23,27-29 The concept of 'insulininduced metabolic stress' has been discussed in more detail previously.15



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**Figure 1.** Model illustrating the molecular basis of insulin-induced metabolic stress in obese insulin-resistant and poorly controlled type 2 diabetes patients. Depicted is a cell in which (a) insulin resistance (IR) protects from nutrient overload and metabolic stress by limiting glucose flux into the cell at times when both glucose and free fatty acids (FFA) are elevated in blood; (b) the IR protection is overridden by a high dose of exogenous insulin therapy which promotes excess glucose uptake and both glucotoxicity and lipotoxicity. High FFA availability inhibits glucose oxidation at the level of pyruvate dehydrogenase (PDH), such that a high glucose flux promoted by high levels of insulin will be forced into glucotoxic pathways above this step, including the polyol and hexosamine pathways, as well as the production of advanced glycation end product (AGE) precursors. Furthermore, high glucose availability promotes build-up of cytosolic malonyl-CoA which will inhibit carnitine palmitoyltransferase 1 (CPT1) and the transfer of long-chain acyl-CoAs (LC-AcylCoA) into mitochondria for  $\beta$ -oxidation. This will result in a push of intracellular FFA metabolism towards synthesis of complex lipids, such as diacylglycerols, cholesterol esters and ceramides, and steatosis causing lipotoxicity. Excess glucose supply to the mitochondria in the presence of high FFA supply will also promote reactive oxygen species (ROS) production and oxidative damage. CD36: free fatty acid transporter; GLUT4: facilitative glucose transporter 4; Ins-R: insulin receptor.

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#### Islet $\beta$ -cell role in obesity and T2D: upstream or downstream of insulin resistance?

The predominant view is that islet  $\beta$ -cell failure in obesityrelated T2D is a consequence of it not being able to sustain high enough insulin secretion to compensate for insulin resistance, suggesting it is downstream and a victim of insulin resistance.<sup>12</sup> However, increasing evidence from pre-clinical and clinical studies support an alternate possibility, at least in subsets of individuals at risk of T2D, that hyper-responsiveness of the islet  $\beta$ -cell to a hostile environment (e.g. from a westernised lifestyle) drives hyperinsulinaemia, this being the culprit and upstream to excessive weight gain, insulin resistance, subsequent  $\beta$ -cell failure and the development of T2D.<sup>30–33</sup>

There is considerable heterogeneity in islet β-cell function in mouse strains with those that have a tendency for insulin hypersecretion (e.g. DBA/2 compared to the C57Bl/6 and 129T2 strains) being more prone to high fat diet-induced weight gain and  $\beta$ -cell failure.<sup>33,34</sup> Furthermore, there are several examples by which suppression of insulin secretion through genetic manipulation can reduce high fat diet-induced obesity and insulin resistance. Islet β-cell-specific deletion of the adipose triglyceride lipase, through reducing the lipid amplification arm of fuelinduced insulin secretion, protects mice from obesity, hyperinsulinaemia, insulin resistance and hyperglycaemia.<sup>35</sup> In addition, through suppressing insulin secretion by knocking out three of the four insulin gene alleles ( $Ins1^{-/-}$ ;  $Ins2^{+/-}$  and  $Ins1^{-/-}$ ;  $Ins2^{+/+}$ ), it has been shown that ageing female mice have lower glycaemia, improved insulin sensitivity and an extended life span.<sup>36</sup> The model less predictably altered insulin secretion in male mice.<sup>30</sup> In the leptin deficient ob/ob mouse model of obesity, a similar genetic approach to lowering insulin secretion, while successfully being able to attenuate obesity, resulted in the development of diabetes, indicative of a need for compensatory hyperinsulinaemia for obesity-related insulin resistance when a rare monogenic cause of obesity rather than hyperinsulinaemia is the primary cause of the excessive weight gain.<sup>37</sup>

Of relevance within human studies is the Da Qing Children Cohort Study which showed that fasting insulin at the age of 5 years, after the adjustment for age, sex, birth weight, TV-viewing time and weight (or body mass index) at baseline, predicted weight gain from age 5 to 10 years.<sup>38</sup> Furthermore, higher insulin levels at 5 years of age were also predictive of higher levels of systolic blood pressure, fasting plasma glucose, insulin resistance as determined by the homeostasis model and triglycerides at 10 years of age, all features of the MetS.<sup>38</sup> The findings were similar to those in a study of Pima Indian children.<sup>39</sup> In addition, adolescent girls with PCOS have been shown to have early-onset insulin hypersecretion in association with insulin resistance.  $^{40}$ 

Pharmacological approaches to suppress insulin secretion in humans also support the view that hyperinsulinaemia may have more of a primary role in the MetS. In obese men, 6 months treatment of lifestyle change with either diazoxide (DZ) alone (inhibits insulin secretion by activating the ATP sensitive potassium channels), DZ with metformin (DZ + M) or placebo showed that DZ (DZ and DZ + M groups combined) markedly reduced fasting insulin levels by 72% compared to only 23% in the placebo group (p < 0.001), and this was accompanied by greater improvements in body weight, LDL cholesterol, HDL cholesterol, triglyceride, and systolic and diastolic blood pressure.41 Similar findings were found when hyperinsulinaemia was suppressed by the somatostatin analogue octreotide LAR in obese subjects, with evidence of responders and non-responders to this therapy.42 Also of relevance, in subjects with T2D, short-term DZ use is capable of restoring islet  $\beta$ -cell function through  $\beta$ -cell rest.<sup>43,44</sup>

Thus, considerable evidence points to insulin hypersecretion as being at, or close to, the root cause of MetS and its related conditions, with insulin resistance being downsteam. Focus on reducing insulin hypersecretion, at least early in the course of these conditions, is likely to have beneficial metabolic effects.

#### Towards better stratification of diabetes: subset of severe insulinresistant and hyperinsulinaemic diabetes

Within a recently reported study of adult-onset diabetes from Scandinavia, five subgroups were identified: severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD) and mild age-related diabetes (MARD).<sup>45</sup>

The subgroup that seems most relevant to this discussion is SIRD, with the predominant characteristics being obesity, severe hyperinsulinaemia and insulin resistance. An alternative name for this subgroup could have been 'severe hyperinsulinaemic diabetes'. Individuals within this subgroup, in keeping with the concept of insulininduced metabolic stress, were also more likely to develop diabetic nephropathy and have coronary events.45 Surprisingly, the age of diabetes onset in the SIRD group was relatively high, which may relate to the predominant Scandinavian ethnicity within the diabetes registries used.<sup>45</sup> The SIRD subgroup characteristics of more severe hyperinsulinaemia and insulin resistance tend to be mirrored in young people presenting with obesity-related T2D, as was found in the Restoring Insulin Secretion (RISE) study and is also reported in various high-risk

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indigenous groups.<sup>40,46,47</sup> T2D in youth is also associated with a much higher risk of early-onset nephropathy and macrovascular disease.<sup>40</sup> If in this subset of diabetes (SIRD), insulin hypersecretion rather than insulin resistance has the primary role, as remains to be determined, it will have major implications on the best approaches to prevention and treatment.

In the SIDD, MOD, and MARD subgroups, insulin resistance is of lesser degree at the time of diabetes diagnosis; however, islet  $\beta$ -cell failure must be involved in the pathogenesis. Whether mild suppression of insulin secretion in at least some of those at risk within these subgroups would prevent this  $\beta$ -cell failure and T2D development is unknown. A precision medicine approach will most likely be required in which the correct approach to diabetes prevention and treatment will require detailed phenotypic and genotypic classification of individual patients within these subgroups.

# A paradigm shift: new conceptual framework for considering insulin resistance and the MetS

If insulin resistance, while clearly being a biomarker of poor metabolic health, is also to be considered a defensive mechanism used by critical tissues against hyperinsulinaemia and nutrient overload, a complete revision of the conceptual framework within which hyperinsulinaemia, insulin resistance and the MetS are viewed, is needed (Figure 2). Such a revision is not trivial, as it has major implications for how MetS and its associated conditions, including T2D, PCOS, NAFLD and ASCVD, should be prevented and managed. Within this new framework and paradigm shift, hyperinsulinaemia has a more primary or causative role. In doing so, instead of the role of the islet  $\beta$ -cell being one of 'compensation' for insulin resistance, it becomes the primary driver, with insulin hypersecretion and the resulting hyperinsulinaemia taking up position as the unifying mechanism. Thus, the development of new therapeutic approaches for MetS, and at least the SIRD subgroup of T2D, will need to move to prevention and/or suppression of the hypersecreting  $\beta$ -cell (Figure 2). Approaches to lower glucose and other elevated nutrients in the blood of MetS and T2D patients through overriding the protective role of insulin resistance will be contraindicated, as we and others have previously advocated.<sup>14–17</sup> While research into mechanisms of islet β-cell failure and insulin resistance will continue to be important, more focus on the mechanisms driving insulin hypersecretion will be required, whether they be genetic or acquired, including those acquired early in life from epigenetic processes and/or consequent on islet  $\beta$ -cell response to new environmental exposures.31

#### **Relevance to management of T2D**

#### Optimisation of cellular nutrient status

In managing disturbed metabolic homeostasis in T2D, the focus of clinicians is currently on normalising glucose and lipid parameters in the blood. Less thought is given to optimising intracellular metabolism, even though nutrientinduced tissue injury in obesity-related T2D is predominantly a consequence of excess entry of nutrients from the blood into cells. This is understandable, as measuring nutrient levels is much easier in blood (e.g. blood glucose, HbA1c and plasma triglycerides) than in cells. The corollary is that approaches to normalise glycaemia in obesity-related T2D that drive glucose and other nutrients into already nutrientoverloaded cells, such as by high-dose insulin therapy or sulphonylureas to override insulin resistance, or insulin sensitisers to reverse insulin resistance depending on mechanism of action, may unintentionally cause harm.<sup>15</sup> According to this argument, alternative approaches to lowering glycaemia that nutrient off-load cells, such as intensive lifestyle measures, sodium-glucose transporter 2 (SGLT2) inhibitors, glucagon-like peptide-1 receptor agonists or bariatric surgery, should be beneficial in the majority of patients with obesity-associated T2D (Figure 3(a)).15

The alternate scenario of intracellular nutrient depletion in patients with hypoinsulinaemic diabetes is also important to consider, particularly, with the increasing occurrence of cases of euglycaemic ketoacidosis in patients treated with SGLT2 inhibitors.<sup>48</sup> Avoidance of SGLT2 inhibitors and most often a shift to insulin therapy will be necessary in such patients (Figure 3(b)).

Thus, the approach to diabetes management should take into account some consideration of cellular nutrient status (Figure 3). For these reasons, new blood biomarkers of cellular nutrient or energy status may also be of value in patient care and once discovered should be examined for clinical utility.

In support of the proposition that glucose-lowering approaches that work by driving glucose into tissues can be harmful in overweight and obese subjects with T2D and insulin resistance, we reviewed major T2D clinical trials and found that whenever intensive glucose-lowering approaches were associated with weight gain of greater than 1.0 kg/year [Action to Control Cadiovascular Risk in Diabetes (ACCORD), Veterans Affairs Diabetes Trial (VADT) and Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction 2 (DIGAMI 2)], cardiovascular and all-cause mortality increased, although only reaching statistical significance in ACCORD given the greater sample size.<sup>15</sup> Furthermore, among adults with diabetes and stable ischaemic heart disease aged  $\geq$ 75 years, insulin provision therapy was associated with an increased risk for all-cause mortality [hazard ratio = 1.89, confidence interval (CI) = 1.1-3.2, p=0.020].<sup>49</sup>



**Figure 2.** A new conceptual framework for considering insulin resistance and the metabolic syndrome (MetS) and its associated disorders. The key changes in this conceptual framework are the roles of hyperinsulinaemia and insulin resistance in the MetS. Islet  $\beta$ -cell hyper-responsiveness to adverse environmental factors in genetically or epigenetically predisposed individuals results in hyperinsulinaemia and this is the primary driver of the MetS. Insulin resistance provides protection for critical tissues against insulin-induced metabolic stress and, while being a biomarker of metabolic ill health, is not a driver of pathogenesis. The characteristic features of MetS are driven by hyperinsulinaemia. The MetS-related disease entities, type 2 diabetes (T2D), cardiovascular diseases (CVD), non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) and polycystic ovary syndrome (PCOS) are a downstream consequence of hyperinsulinaemia and the MetS.

In support of the benefits of nutrient off-loading approaches are more recent clinical trials of new classes of glucose-lowering agents, such as SGLT2 inhibitors (by promoting urinary glucose loss) and GLP-1 receptor agonists (by reducing weight through increased satiety), as well as bariatric surgery that have demonstrated reductions in major adverse cardiovascular and renal outcomes in high-risk T2D patients.<sup>50–52</sup> The recent consensus statement of the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) on the management of hyperglycaemia in T2D has taken the results of these major clinical trials into consideration in their recommendations.<sup>53</sup>

#### Prevention of insulin hypersecretion

The nutrient off-loading approaches to glucose lowering available in the management of T2D, including intensive lifestyle change, SGLT2 inhibitors, GLP-1 receptor antagonists,  $\alpha$ -glucosidase inhibitors and bariatric surgery, will all reduce insulin hypersecretion. However, often these therapies are started once T2D is established and failure of islet

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**Figure 3.** Optimisation of cellular nutrient status in patients with hyper- or hypoinsulinaemic type 2 diabetes: importance of the approach to glucose lowering. (a) In untreated type 2 diabetes (T2D) with hyperinsulinaemic diabetes, insulin resistance (IR) protects insulin-responsive cells such as cardiomyocytes and skeletal muscle cells from nutrient overload; cells such as endothelial cells that are non-responsive to insulin with respect to glucose uptake, however, are not protected and are injured by glucotoxicity contributing to diabetes complications (left panel). Glucose-lowering approaches that override the physiological IR to force glucose into insulin-responsive tissues (e.g. by high-dose insulin therapy) may reduce glucotoxicty in some tissues, but at the cost of nutrient-induced injury to the insulin-responsive tissues (e.g. causing a metabolic cardiomyopathy) (centre panel). Glucose-lowering approaches that off-load glucose from cells of critical body tissues,

(Continued)

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#### Figure 3. (Continued)

by either reducing glucose entry into the blood (e.g. intensive lifestyle,  $\alpha$ -glucosidase inhibitors and bariatric surgery) or glucose clearance from the blood via non-toxic routes (e.g. SGLT2 inhibitors to promote urinary glucose loss, exercise and activation of brown adipose tissue), will reduce nutrient-induced tissue injury in all cell types (right panel). (b) In untreated hypoinsulinaemic T2D, a starvation response to insulin deprivation occurs in which free fatty acid (FFA) release from adipose tissue is increased and hepatic ketone body production is increased; cardiomyocytes and skeletal muscle cells are starved of glucose and switch to use FFA and ketone body for energy; cells such as endothelial cells are not protected from hyperglycaemia (left panel). In this circumstance, insulin therapy will prevent the starvation response and normalise cellular nutrient status in insulin-responsive tissues and prevent glucotoxicity in endothelial cells (centre panel). Glucose-lowering approaches that off-load glucose from cells (e.g. by very low carbohydrate diets or fasting for surgery) together with glucose clearance from the blood (e.g. SGLT2 inhibitors) will exacerbate the starvation response in hypoinsulinaemic T2D and induce euglycaemic ketoacidosis (right panel). Healthy cells are shown in green; unhealthy nutrient-overloaded cells are shown in red; and unhealthy nutrient-deprived cells are shown in blue.

β-cells has already commenced. Optimal approaches for reversal of severe hyperinsulinaemia in patients prior to development of T2D or early in its course, in particular in younger individuals, when lifestyle measures are generally unsuccessful, are not known. Of note, bariatric surgery has been shown to be effective in reversing hyperinsulinaemia and MetS in obese adolescents.<sup>54</sup> In the RISE study, neither 3 months of insulin glargine followed by 9 months of metformin nor 12 months of metformin alone slowed the progressive deterioration of β-cell function in young people with early T2D, suggesting different approaches are required.<sup>55</sup> The development of specific islet β-cell therapies to limit insulin hypersecretion in high-risk individuals with MetS-related conditions and obesity-related pre-diabetes and early T2D should be pursued.

#### Conclusion

The part played by Jerry Reaven in linking the dots between the various components of the MetS and the relevance of MetS to ASCVD, NAFLD, PCOS and T2D has been enormously important. The search for the unifying mechanism has been contentious. Here, we make a case for putting 'insulin hypersecretion' into this role, while considering insulin resistance as a protective downstream response. This necessitates a complete revision of the conceptual framework within which we view insulin resistance and the pathophysiology of the MetS and obesity-associated T2D, which if confirmed, has major implications for the prevention and management of these metabolic conditions.

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# Insulin resistance: Impact on therapeutic developments in diabetes

#### **Clifford J Bailey**

#### Abstract

Insulin resistance has a broad pathogenic impact affecting metabolic, cardio-renal and other disease areas. Extensive studies to dissect the mechanisms of insulin resistance have provided valuable insights to shape current clinical awareness and advance therapeutic practice. However, the development of direct interventions against insulin resistance has been hindered by its complex and highly variable presentations, especially in type 2 diabetes. Among glucose-lowering agents, metformin and thiazolidinediones provide cellular actions that counter some effects of insulin resistance: reduced glucotoxicity and weight-lowering with antidiabetic therapies also improve insulin action, except that endogenously-or exogenously-created hyperinsulinaemia may partially compromise these benefits. Increasing awareness of the pervasiveness and damaging ramifications of insulin resistance heightens the need for more specifically targeted and more effective therapies.

#### **Keywords**

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Insulin resistance, diabetes therapies, metformin, sulfonylureas, glucagon-like peptide-1 receptor agonists, insulin, thiazolidinediones, dipeptidyl peptidase-4 inhibitors

#### Introduction

This short review is written in recognition of the seminal works of Gerald Reaven on the role of insulin resistance in the pathogenesis of type 2 diabetes and cardiovascular (CV) disease. Foregoing reviews in this issue of *Diabetes and Vascular Disease Research* have eminently recounted these works.<sup>1-4</sup> Here, we consider how a growing appreciation of insulin resistance influenced the development of new therapeutics in the field of diabetes.

#### Multifactorial pathophysiology

Reaven's Syndrome X (not to be confused with the cardiac syndrome X) describes the clustering of CV risk factors that depend on or associate with insulin resistance.<sup>5</sup> Although Reaven's<sup>6,7</sup> Syndrome X later became subsumed within the so-called Metabolic Syndrome, the two syndromes are not synonymous: insulin resistance can promote CV disease independent of other CV risk factors commonly included in the Metabolic Syndrome such as excess adiposity, and insulin resistance is often associated with compensatory hyperinsulinaemia, at least in its early pathogenesis, which further aggravates metabolic, vascular and haemodynamic disturbances. Studies on the aetiology of insulin resistance and accompanying metabolic and CV abnormalities gave rise to a 'common soil' hypothesis of shared origins, and clinical practice recognised that the

presence of one feature associated with insulin resistance should prompt suspicion about other CV risk factors.<sup>8</sup> This in turn has promoted a more holistic multifactorial approach to the assessment and management of type 2 diabetes to accommodate metabolic and cardio-renal aspects.<sup>9,10</sup>

Insulin resistance presents across a wide variety of phenotypes with different responses between tissues, organs and physiological systems. Because this is typically due to multiple 'bottle-necks' in the signalling pathways that mediate the biological actions of insulin, it has not been possible to identify a single cure-all molecular target.<sup>2</sup> However, several glucose-lowering agents can ameliorate the effects of insulin resistance with impact on diabetic complications, particularly in type 2 diabetes (Figure 1).

#### Insulin

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When Harold Himsworth described insulin resistance in the 1930s, he energised debate about different types of diabetes.<sup>11</sup> Studies by Yalow and Berson<sup>12</sup> in the late 1950s noted that insulin concentrations might actually be higher

Corresponding author:

Clifford J Bailey, School of Life & Health Sciences, Aston University, Birmingham B4 7ET, UK. Email: c.j.bailey@aston.ac.uk

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School of Life & Health Sciences, Aston University, Birmingham, UK



Figure 1. Timeline of some key events regarding insulin resistance and the introduction of diabetes therapies. Metformin was introduced in the United States in 1995.

CSII: continuous subcutaneous insulin infusion; CV: cardiovascular; DPP-4is: dipeptidyl peptidase-4 inhibitors; GLP-1RAs: glucagon-like peptide-1 receptor agonists; PCOS: polycystic ovary syndrome; SGLT-2is: sodium/glucose co-transporter-2 inhibitors; TZDs: thiazolidinediones.

in the early stages of 'maturity-onset diabetes', which substantiated the Himsworth premise. Indeed, excess insulin has been mooted as a possible atherogenic factor,<sup>13</sup> and concern about the use of high doses of insulin therapy was highlighted by evidence that raised insulin concentrations do not rectify insulin resistance and may lead to an increasing spiral of insulin demand through further disruption to insulin receptor binding and post-receptor signalling.<sup>14</sup> Thus, an appreciation of Syndrome X helped to redirect attention towards sparing insulin rather than increasing insulin, particularly in the earlier stages of type 2 diabetes.

While this illustrates the rationale for changing the management focus of type 2 diabetes beyond insulin, the main alternative up to the 1990s was the use of sulfonylureas which act mostly by stimulating insulin secretion.

#### **Sulfonylureas**

The first sulfonylureas (e.g. carbutamide and tolbutamide) from the mid 1950s and more potent versions from the mid 1960s (e.g. glibenclamide) have well-studied glucose-lowering properties in type 2 diabetes, but incur weight gain and risk of hypoglycaemia.<sup>15</sup> However, the effects of sulfonylureas on insulin resistance have not been consistent, and CV effects are also unclear. The much criticised University Group Diabetes Programme (UGDP) cast doubt on the CV safety of tolbutamide in the late 1960s,<sup>16</sup> but the United Kingdom Prospective Diabetes Study

(UKPDS)<sup>17,18</sup> and many other trials have shown a better CV prognosis with sulfonylureas than diet/lifestyle but less beneficial than metformin. Similarly, the effects of meglitinides on insulin resistance and CV events remain unclear but appear minimal. Nevertheless, the detrimental impact of hypoglycaemia on CV events and the adverse effects of insulin resistance on islet beta-cell function must be considered in selecting glucose-lowering therapies.<sup>19,20</sup>

#### **Biguanides**

Although several guanidine derivatives were used in the treatment of diabetes in the 1920s and 1930s, their use dwindled as insulin became available, and it was not until the late 1950s that three biguanides (metformin, phenformin and buformin) were introduced in Europe and one (phenformin) was introduced in the United States.<sup>21</sup> Phenformin and buformin were withdrawn in the late 1970s due to high risk of lactic acidosis, and metformin was eventually introduced in the United States in 1995. Since biguanides lower blood glucose in type 2 diabetes without stimulating insulin secretion, it was acknowledged that they counter insulin resistance, and this was initially attributed to increased anaerobic metabolism and an independent reduction of hepatic gluconeogenesis. Lack of weight gain and low risk of hypoglycaemia favoured early use of metformin in type 2 diabetes, supported by mounting evidence for long-term reductions in CV disease.18,22 Through

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increased appreciation of the pathogenic effects of insulin resistance, Reaven's studies contributed an important part of the scientific platform for metformin and its present-day position as first-line pharmacological therapy for type 2 diabetes. Reaven's group also conducted several key studies on the mode of action of metformin, for example, showing the inter-relationship of effects on glucose and lipid homeostasis.<sup>23</sup> The group also noted that metformin prolongs insulin receptor tyrosine kinase activity.<sup>24</sup>

#### Thiazolidinediones

Thiazolidinediones (TZDs) emerged from lipid-lowering clofibrate analogues in the mid 1970s, before peroxisome proliferator-activated receptor (PPAR) molecules had been discovered, but it was not until the late 1990s that the first PPARY agonist (troglitazone) was introduced and then withdrawn due to unexplained hepatotoxicity. Rosiglitazone and pioglitazone followed promptly: they lowered plasma glucose without raising insulin, mostly through genomic effects that include differentiation of new small insulin-sensitive subcutaneous adipocytes, and improved insulin action in liver and muscle.<sup>25</sup> Pioglitazone also has some PPARa agonism which assists lipid control, but weight gain associated with adipose deposition and renal effects to increase fluid retention and risk of heart failure limited their use. Rosiglitazone was withdrawn in Europe in 2010 amid controversy over possible adverse CV effects, and potential risk of bone fractures has further limited use. Although TZDs provided an antidote to insulin resistance, their limitations illustrate the complexities and ambiguities of increasing insulin action across a breadth of biological functions without modulating effects in different tissues.26

#### Incretins

Emanating from studies of the entero-insular axis, the availability of glucagon-like peptide-1 receptor agonists (GLP-1RAs) from 2005 and dipeptidyl peptidase-4 (DPP4) inhibitors from 2006 shifted the therapeutic focus of type 2 diabetes back to the pancreas. These agents do not carry the risk of hypoglycaemia seen with sulfonylureas because they potentiate insulin secretion and suppress glucagon secretion in a glucose-dependent manner (sulfonylureas stimulate insulin secretion independently of the glucose concentration).<sup>27,28</sup> Interestingly, GLP-1 RAs and DPP4 inhibitors reduce insulin resistance: this appears to be due, at least in part, to a lowering of glucose concentrations, interrupting the vicious spiral of type 2 diabetes in which insulin resistance generates hyperglycaemia and the ensuing glucotoxicity aggravates insulin resistance.20 The satiety effect of GLP-1RAs, which is associated with weight loss and decreased adiposity, provides further metabolic and endocrine mechanisms to reduce insulin resistance, and potential additional incretin-based therapies including peptide YY (PYY), oxyntomodulin, derivatives of gastric inhibitory polypeptide (GIP) and antagonists of ghrelin are under investigation.<sup>29</sup>

## Sodium/glucose co-transporter-2 inhibitors

Sodium/glucose co-transporter-2 (SGLT2) inhibitors introduced in 2012 reduce glucotoxicity and adiposity by eliminating excess glucose in the urine and thereby act indirectly to reduce insulin resistance and spare some of the demand on beta-cell function.<sup>30,31</sup> Further evidence that lowering blood glucose will in turn lower insulin resistance is provided by alpha-glucosidase inhibitors which reduce prandial glucose excursions by slowing the rate of carbohydrate digestion.

# Adipose and anti-obesity agents to reduce weight

Excess lipids, endocrine factors and pro-inflammatory molecules from adipose tissue are well known to promote the pathogenesis of insulin resistance in obese type 2 diabetes, and several adipokine-based therapies such as adiponectin receptor agonists are receiving consideration as potential approaches to counter insulin resistance. Improvements of insulin action and glycaemic control are consistently reported with caloric restriction and reduced adipose mass (particularly in omental, hepatic and pancreatic locations), whether achieved by dieting, bariatric procedures, SGLT2 inhibitors, GLP-1RAs or other appetite/satiety-modifying therapies.<sup>32,33</sup> We may wonder why the age-old energy-reducing approach to treating obese-diabetes has taken so long to regain prominence.

#### CV and other considerations

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It is perhaps an irony that one of the TZDs (rosiglitazone), which improved insulin sensitivity and reduced a range of atherogenic risk markers, should have triggered CV safety concerns and prompted current regulatory requirements for specific and extensive evaluation of CV events with new glucose-lowering agents.<sup>26</sup> Although there are many unanswered questions regarding the subtle interplay of insulin resistance and hyperinsulinaemia on the endothelium and myocardium, it is evident that early intervention to achieve long-term metabolic control and reduce insulin resistance provides an opportunity to improve CV prognosis.<sup>18,26</sup> Timing appears to be especially important in this context because late interventions have been less successful against CV disease. The early development of hyperinsulinaemia with insulin resistance may promote hyperfiltration and damage to glomeruli, and insulin resistance is implicated in a wide variety of conditions including

polycystic ovary syndrome and dementia indicating the breadth of potential benefits to be gained from effective timely interventions.<sup>1–4,34,35</sup>

#### Future

Awareness of insulin resistance as an underlying and modifiable pathogenic factor spanning diabetes, CV and other disease areas makes it an important therapeutic target. However, despite considerable appreciation of insulinreceptor interactions and post-receptor signalling, therapeutic interventions have been unable to rectify or circumvent the complex multi-dimensional defects of insulin resistance.35 Several current therapies do act, at least in part, to address the metabolic disturbances and provide some protection against adverse CV events associated with type 2 diabetes, but it is unclear how these interventions will impact other disease areas susceptible to insulin resistance. New therapeutic approaches, including small non-peptide molecules that partially mimic insulin effects at the insulin receptor or initiate or potentiate receptor tyrosine kinase activity or target post-receptor pathways, have been identified, but these are still at early stages of investigation.<sup>36</sup> Thus, the therapeutic reversal of insulin resistance seems destined to be an ongoing unmet need for the foreseeable future.

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# **Pioglitazone: The forgotten, cost-effective cardioprotective drug for type 2 diabetes**

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Ralph A DeFronzo<sup>1</sup>, Silvio Inzucchi<sup>2</sup>, Muhammad Abdul-Ghani<sup>1</sup> and Steven E Nissen<sup>3</sup>

#### Abstract

Type 2 diabetes individuals are at high risk for macrovascular complications: myocardial infarction, stroke and cardiovascular mortality. Recent cardiovascular outcome trials have demonstrated that agents in two antidiabetic classes (SGLT2 inhibitors and GLP-I receptor agonists) reduce major adverse cardiovascular events. However, there is strong evidence that an older and now generically available medication, the thiazolidinedione, pioglitazone, can retard the atherosclerotic process (PERISCOPE and Chicago) and reduce cardiovascular events in large randomized prospective cardiovascular outcome trials (IRIS and PROactive). Pioglitazone is a potent insulin sensitizer, preserves beta-cell function, causes durable reduction in HbA1c, corrects multiple components of metabolic syndrome and improves nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. Adverse effects (weight gain, fluid retention, fractures) must be considered, but are diminished with lower doses and are arguably outweighed by these multiple benefits. With healthcare expenses attributable to diabetes increasing rapidly, this cost-effective drug requires reconsideration in the therapeutic armamentarium for the disease.

#### **Keywords**

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Pioglitazone, type 2 diabetes mellitus, cardiovascular disease

#### Background

Type 2 diabetes mellitus (T2DM) is a cardiometabolic disease<sup>1,2</sup> that affects both the microvasculature (retinopathy, nephropathy, neuropathy) and macrovasculature [myocardial infarction (MI), stroke]. The microvascular complications primarily are related to the level of glycaemic control,<sup>3,4</sup> whereas hyperglycaemia is a relatively weak risk factor for the macrovascular complications<sup>3,5</sup> which represent the major cause of mortality in T2DM patients.<sup>6,7</sup> Long-term cardiovascular (CV) outcome trials have generally demonstrated no or only slight reduction in CV events with intensive glycaemic control.<sup>3,8–10</sup> In contrast, treatment of more traditional CV risk factors (blood pressure, dyslipidaemia) consistently has been associated with major CV benefits in T2DM patients.<sup>1</sup>

The results of recent CV outcome trials have documented that glucose-lowering agents in two different classes significantly reduce the MACE (major adverse cardiovascular events) endpoint (composite of CV mortality, non-fatal MI, non-fatal stroke). In both the EMPA REG OUTCOME trial<sup>11</sup> and in the CANVAS program,<sup>12</sup> the sodium glucose transporter-2 (SGLT2) inhibitors, empagliflozin and canagliflozin, reduced MACE by 14% and 13%, respectively, although the relative contributions of the three individual components of the composite outcome differed. In LEADER<sup>13</sup> and SUSTAIN-6,<sup>14</sup> therapy with the glucagon-like-peptide receptor agonists (GLP-1 RAs), liraglutide and semaglutide, resulted in reductions in MACE of 13% and 26%, respectively, and also with differential contributions from the composite elements. Importantly, empagliflozin and liraglutide were each associated with significant reductions in CV mortality as well. With the robust results of these large, long-term, CV outcome trials, we are entering a new era of T2DM treatment where glucose-lowering drugs that address both glycaemia, as well as CV risk, are now preferred in patients with cardiovascular disease (CVD) over those therapies that simply lower HbA1c.<sup>15</sup>

In the midst of the newfound interest in the SGLT2 inhibitors and GLP-1 RAs, the established anti-atherogenic benefits of the thiazolidinedione (TZD), pioglitazone, have been overlooked.<sup>16</sup> The recent results of the IRIS (Insulin Resistance Intervention after Stroke) trial<sup>17</sup>

#### **Corresponding author:**

Ralph A DeFronzo, Division of Diabetes, Department of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA. Email: albarado@uthscsa.edu

<sup>&</sup>lt;sup>1</sup>Division of Diabetes, Department of Medicine, University of Texas Health Science Center, San Antonio, TX, USA <sup>2</sup>Endocrine Division, Yale School of Medicine, New Haven, CT, USA <sup>3</sup>Cleveland Clinic Foundation, Cleveland, OH, USA

should rekindle interest in pioglitazone as a cardioprotective drug, an effect which actually was established more than a decade ago. Because pioglitazone is now generically available, it represents a more affordable option than either an SGLT2 inhibitor or a GLP-1 RA.<sup>18</sup> Furthermore, it can be combined with these and other glucose-lowering agents, including the SGLT2 inhibitors or GLP-1 RAs, to minimize side effects.<sup>15,18-20</sup> Pioglitazone also has a number of other demonstrated benefits, including amelioration of insulin resistance, preservation of beta-cell function, durable glycaemic control, improvement of multiple factors of the metabolic syndrome and reversal of hepatic steatosis [nonalcoholic fatty liver disease (NAFLD)]/nonalcoholic steatohepatitis (NASH) making it an attractive option for the treatment of many patients with T2DM, particularly those at risk for CV events. In this review, we examine the CV, glycaemic and other metabolic benefits of pioglitazone and provide strategies to maximize the drug's benefit: risk ratio.

#### **CV** benefit

A substantial body of evidence, including large randomized prospective CV outcome trials,16,17,21-23 real-world observational studies24-26 and smaller studies of regression of coronary atherosclerosis27 and carotid intima thickness,28 has demonstrated that pioglitazone reduces both atheroma progression and related CV events. These investigations were initiated because of a substantial literature dating back many decades that has linked insulin resistance to premature coronary heart disease (CHD).<sup>29</sup> These data begged the question as to whether reducing insulin resistance with an insulin-sensitizing drug would provide a CV benefit. The question is also of historical importance, since the notion that a diabetes drug could reduce CV events had eluded investigators for years. In PROactive, 5238 T2DM patients with a prior CV event were randomized to pioglitazone or placebo and followed for a mean of 2.9 years.<sup>16</sup> Although the primary endpoint, a broad composite that included leg revascularization procedures, fell short of statistical significance [hazard ratio (HR)=0.90, p=0.09], the 'main secondary endpoint', MACE, was significantly reduced (HR=0.84, p=0.027) (Figure 1), on par with the effect size in the aforementioned recent positive trials of newer glucose-lowering agents.<sup>11-15</sup> In PROactive participants with a prior MI (n=2445) or prior stroke (n=948) pioglitazone therapy were associated with robust 28% and 47% reductions in recurrent MI<sup>30</sup> and recurrent stroke,<sup>31</sup> respectively. The primary endpoint in PROactive<sup>16</sup> should be interpreted in the context that leg revascularization historically has not been included as an endpoint in CV outcome trials since it is refractory to antihypertensive, lipid-lowering and glucose-lowering therapy.<sup>32,33</sup> Consistent with PROactive, a meta-analysis of published pioglitazone studies and reported to the Food and Drug Administration (FDA) demonstrated a 25% reduction in CV events.<sup>21,22</sup>

Based upon (1) evidence that insulin resistance was a strong risk factor for stroke as well CHD,<sup>2</sup> (2) the consistently positive results observed in these CV outcome trials,<sup>16,17,21,22,24–28</sup> and (3) the reduction in recurrent stroke (by 47%) and MI (by 28%) in T2DM individuals in PROactive,<sup>16</sup> the National Institutes of Health initiated the IRIS study.17 In 3876 non-diabetic, insulin-resistant individuals with a recent transient ischaemic attack (TIA) or stroke, pioglitazone reduced fatal/non-fatal stroke or MI by 24% (p=0.007) over a mean of 4.8 years (Figure 2).<sup>17</sup> In a follow-up report from this study,<sup>34</sup> pioglitazone reduced the risk of any stroke by 25% (p=0.01) and decreased the risk of acute coronary syndrome by 29% (p=0.02), with most of the drug's effects on type 1 MIs (HR = 0.62, p = 0.03), particularly large infarcts (HR = 0.44, p=0.02).<sup>35</sup> These results compare favourably with results obtained with aspirin and anti-platelet drugs, 36-38 as well as with statins,<sup>39</sup> which are now widely used for stroke prevention.<sup>36–38</sup> Notably, the positive beneficial CV effects of pioglitazone in all of these studies occurred on the background of widespread use of evidence-based CV therapies including anti-platelet agents suggesting that pioglitazone can effectively address 'residual CV risk'.

Observational 'real-world' data also support the CV benefits of pioglitazone. For example, a retrospective analysis of 91,511 patients in the UK Research General Practice Database (GPRD) who were followed for 7.1 years demonstrated that pioglitazone decreased allcause mortality by 39% compared with metformin.40 In a separate analysis of 27,457 GPRD patients who had a second agent added to metformin monotherapy, pioglitazone therapy was associated with a significantly decreased HR for all-cause mortality (HR=0.71) and the combined endpoint of all-cause mortality/major adverse CV events (HR=0.75).<sup>24</sup> In a more recent observational study,<sup>25</sup> pioglitazone significantly reduced both CV (HR=0.58) and non-CV (HR=0.63) mortality in a large (n=62,266)European cohort of diabetic patients. In a study which compared 56,536 patients with T2DM who were firsttime users of pioglitazone or insulin, propensity scores showed a 67% reduction in all-cause mortality in favour of pioglitazone.<sup>26</sup> In a meta-analysis of nine randomized controlled trials, pioglitazone significantly reduced the risk of major CV events in patients with diabetes [HR=0.83, 95% confidence interval (CI)=0.72-0.97] and prediabetes or insulin resistance (HR=0.77, 95% CI=0.64-0.93).<sup>41</sup> The results of this meta-analysis are consistent with a previous one by Lincoff et al.<sup>21</sup> Finally, consistent with the IRIS study, another retrospective study from the UK using Clinical Practice Research Datalink (CPRD) found a HR of 0.63 for incident stroke in T2DM patients who were users of pioglitazone versus other glucose-lowering drugs.42



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**Figure I.** (a) Kaplan–Meier plot of time to MACE endpoint (cardiovascular mortality, non-fatal MI, non-fatal stroke) in T2DM patients treated with pioglitazone (PIO) or placebo (PIc) in PROactive. Redrawn with permission from Dormandy et al.<sup>16</sup> (b) Pioglitazone reduces recurrent MI in diabetic patients with a previous MI in PROactive. Redrawn with permission from Erdmann et al.<sup>30</sup> (c) Pioglitazone reduces recurrent stroke in diabetic patients with a previous stroke in PROactive. Redrawn with permission from Wilcox et al.<sup>31</sup> (d) Meta-analysis of all published studies (excluding PROactive) in which the effect of pioglitazone versus placebo or active comparator on cardiovascular events is examined. Redrawn with permission from Lincoff et al.<sup>21</sup>

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**Figure 2.** Effect of pioglitazone versus placebo on recurrent stroke and myocardial infarction in the Insulin Resistance Intervention after Stroke (IRIS) study. Drawn from the data in Kernan et al.<sup>17</sup>

Smaller mechanistic studies are consistent with the findings from these large prospective and observational studies and meta-analyses. In the PERISCOPE study, pioglitazone, compared with glimepiride, retarded the progression of coronary atherosclerosis as measured by intravascular ultrasound (IVUS),<sup>27</sup> while in the CHICAGO study, pioglitazone slowed the rate of increase in carotid intimal thickness, a surrogate measure of atherosclerosis.<sup>28</sup> Pioglitazone has been shown to reduce intracoronary plaque volume in non-diabetic<sup>43</sup> and type 2 diabetic<sup>44</sup> subjects and to prevent restenosis after stent placement.<sup>45</sup>

One negative pioglitazone study to consider is the recent CV outcome trial from Italy, TOSCA-IT.46 A total of 3041 T2DM patients with suboptimal glycaemic control on metformin monotherapy were randomized to either pioglitazone or a sulphonylurea and followed for a mean of 4.8 years. Because only 11% had a prior history of CVD, this was essentially a primary prevention population. The primary outcome (all-cause death, non-fatal MI, non-fatal stroke and urgent coronary revascularization) occurred at a similar frequency between the two groups: pioglitazone 6.8% versus sulphonylurea 7.2% (HR=0.96, p=0.40). Unfortunately, the study had some methodological limitations, including its unblinded design and the fact that many patients in the pioglitazone arm had either terminated their participation early (10%) or had stopped the study drug (28%), likely stemming from controversy about the drug's safety that had arisen during the trial. Furthermore, the CV event rate, 1.5 per 100 person years, was very low, rendering the study greatly underpowered to detect any effective CV events. This issue was underscored by an a posteriori per-protocol analysis focusing on just those patients taking

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Table 1. Effect of pioglitazone on established CV risk factors.

CV risk factors	Effect of pioglitazone			
Obesity (visceral)	Improves – redistributes fatª			
Hypertension	Decreases BP			
Hypertriglyceridaemia	Decreases TG			
Low HDL cholesterol	Increases HDL			
Small dense LDL particles	Converts to larger more buoyant LDL			
Endothelial dysfunction	Improves			
Hyperglycaemia	Durable decrease in HAIc			
Inflammation (hsCRP)	Reduces			
Lipotoxicity	Reverses			
NASH/NAFLD	Improves			
PAI-I	Reduces			
Hyperinsulinaemia	Decreases			
Insulin resistance	Improves			

CV: cardiovascular; BP: blood pressure; TG: triglyceride; HDL: highdensity lipoprotein; LDL: low-density lipoprotein; hsCRP: high-sensitive C-reactive protein; NASH/NAFLD: nonalcoholic steatohepatitis/nonalcoholic fatty liver disease.

<sup>a</sup>Although subjects treated with pioglitazone may gain weight, visceral, hepatic and muscle fat content are decreased.

their study drug. Here, a secondary outcome that included peripheral vascular events was significantly reduced by study drug (HR=0.67, p=0.03). Of course, this outcome must be interpreted cautiously.

In summary, a large body of evidence from clinical trials to observational studies to mechanistic investigations document that pioglitazone effectively prevents recurrent CV events in both diabetic and non-diabetic individuals, most likely through a beneficial effect on atherosclerosis. The only negative study to our knowledge, TOSCA-IT, involved patients generally without CVD and had multiple interpretative challenges.

Appropriate concern has been raised about 'heart failure' (HF) in PROactive. Due to increased renal sodium retention, all TZDs are associated with oedema,<sup>47</sup> which is a nonspecific sign of HF by clinicians. Since the HF cases in PROactive were not adjudicated, it remains possible that at least some of the excess events may have reflected cases of oedema without cardiac decompensation. HF is typically an ominous diagnosis in patients with diabetes, with a 5-year mortality in excess of 50%.<sup>1,45</sup> Given that mortality in this cohort of individuals with HF in PROactive was decreased (not increased), albeit not significantly, it is possible even likely that not all (probably many) patients diagnosed with HF actually had HF. In fact, in smaller trials, pioglitazone has been demonstrated to have no deleterious effect on left ventricular (LV) function,48,49 to actually improve diastolic dysfunction,48-50 to reduce blood pressure,48,49 and to increase myocardial insulin sensitivity.48,49 Interestingly, prior to the concern about HF, observational data suggested that this drug class might actually decrease mortality after HF admissions.<sup>16</sup> Thus, participants in PROactive who were diagnosed with HF did not experience any increase in CV events compared to placebo-treated individuals.<sup>16</sup> In IRIS, HF was not increased, although this cohort of cerebrovascular patients had less CHD than did participants in PROactive.<sup>17,51</sup> Also, the IRIS protocol allowed for dose reductions in the setting of significant weight gain or oedema. These observations are consistent with previous findings that pioglitazone improves diastolic function in diabetic rats<sup>52</sup> and humans<sup>48,53</sup> by positively influencing matrix remodelling.<sup>52,53</sup> A recent meta-analysis suggests that pioglitazone also reduces both new onset and recurrent atrial fibrillation by 27%.<sup>54</sup>

#### Metabolic effects of pioglitazone

The insulin resistance syndrome (IRS), originally referred to as the metabolic syndrome, comprises a cluster of cardiometabolic disorders, each representing an independent CV risk factor.<sup>2</sup> Pioglitazone improves each component of the IRS (Table 1) (reviewed in previous studies<sup>2,55-58</sup>). It enhances insulin sensitivity and effectively reduces plasma glucose levels and HbA1c while also lowering blood pressure and having a favourable effect on the plasma lipid profile. The latter includes a reduction in triglycerides and free fatty acids (FFAs), increase in high-density lipoprotein (HDL) cholesterol and conversion of small dense low-density lipoprotein (LDL) particles to larger, more buoyant, less atherogenic ones. The drug also shifts fat from visceral abdominal depots, from liver and from skeletal muscle to subcutaneous abdominal depots,59-62 thereby ameliorating lipotoxicity.2,55,63-66 It normalizes adipocytokine secretion, especially adiponectin, improves endothelial dysfunction and reduces circulating concentrations of the procoagulant plasminogen activator inhibitor-1 and the pro-inflammatory mediator C-reactive protein (CRP).66,67 Although pioglitazone improves multiple CV risk factors, both preclinical<sup>68-70</sup> and clinical<sup>16,17,21-23,27,28,71</sup>, data suggest that pioglitazone exerts direct anti-atherogenic effects on the arterial wall.

Pioglitazone transacts its effects through activation of the nuclear hormone receptor peroxisome proliferator– activated receptor-gamma (PPAR $\gamma$ ).<sup>72</sup> PPAR $\gamma$  receptors are expressed in endothelial cells, arterial smooth muscle cells and monocytes/macrophages, providing a pathway for direct anti-inflammatory, antioxidant and other protective actions of pioglitazone.<sup>73–77</sup> Pioglitazone is the only true insulin-sensitizing antidiabetic agent<sup>78</sup> and insulin resistance has been independently associated with atherosclerotic CVD in many cross-sectional and prospective studies.<sup>2,29,58,79–84</sup>

### Pioglitazone is a potent insulin sensitizer

The core pathophysiologic defects in T2DM are insulin resistance in muscle and liver and beta-cell failure.<sup>2,55,85</sup>

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Collectively, these three pathophysiologic disturbances have been termed the TRIUMVIRATE.2,55 Insulin resistance in liver results in excess glucose production during the sleeping hours and is the primary factor responsible for fasting hyperglycaemia, while insulin resistance in muscle is the primary factor responsible for postprandial hyperglycaemia. Impaired suppression of hepatic glucose production and reduced liver glucose uptake following a meal also contribute to the postprandial hyperglycaemia.55,85 Progressive beta-cell failure<sup>55,85-88</sup> accentuates the insulin resistance in liver and muscle. In addition, the adipocyte is resistant to insulin,<sup>2,55,85</sup> resulting in accelerated lipolysis and increased circulating plasma FFA concentrations.89,90 Elevated plasma FFA in turn exacerbate the muscle insulin resistance,91 stimulate hepatic gluconeogenesis and inhibit hepatic glucose uptake92 and impair beta-cell function.93 Pioglitazone improves insulin sensitivity in skeletal<sup>2,55,72,94-96</sup> and cardiac<sup>48,49</sup> muscle, in liver<sup>97</sup> and in adipose tissue98 via multiple mechanisms: PPARy activation, stimulation of the insulin signal transduction system, improved glucose transport/glycogen synthesis/glucose oxidation, increased mitochondrial function, reduced plasma FFA levels and reversal of lipotoxicity.2,55,63-66

## Pioglitazone improves beta-cell function

Insulin resistance is the earliest detectable disturbance in the natural history of T2DM.<sup>2,55,85</sup> However, overt diabetes does not develop in the absence of beta-cell failure and progressive decline in insulin secretion.<sup>2,55,85-88</sup> Although not well appreciated, TZDs, including pioglitazone, in addition to their insulin-sensitizing action, exert a potent effect to preserve beta-cell function99,100 and durability of glycaemic control has been demonstrated in eight longterm, double-blind, placebo-controlled or active comparator studies for up to 5 years (reviewed by DeFronzo<sup>55</sup>). Multiple studies performed in subjects with impaired glucose tolerance (IGT) also have demonstrated a potent action of TZDs to augment beta-cell function (reviewed in previous studies<sup>86,100,101</sup>). For example, in the ACT NOW study,100,102 conversion of IGT to T2DM was reduced by 72% and improvement in the insulin secretion/insulin resistance (disposition) index (gold standard measure of beta-cell function) was the strongest predictor of diabetes prevention.<sup>100</sup> The improvement in beta-cell function is related to stimulation of PPARy receptors on the beta cell, enhanced beta-cell sensitivity to glucose and reversal of lipotoxicity.55,103

#### **Pioglitazone improves NASH/NAFLD**

NAFLD has reached epidemic proportions in the United States and worldwide<sup>104</sup> and is the precursor for NASH.<sup>105</sup> Diabetic patients with NASH are at high risk for cirrhosis and hepatocellular carcinoma.<sup>106</sup> Patients with NAFLD/

NASH are markedly resistant to insulin, often have the metabolic syndrome and are also at increased risk for CVD.<sup>107–110</sup> Because pioglitazone improves insulin sensitivity, corrects multiple components of the IRS, ameliorates lipotoxicity and protects against atherosclerotic CVD, it would be an excellent agent for the treatment of NAFLD and NASH. Indeed, multiple studies have demonstrated that pioglitazone consistently reduces hepatic fat content and reverses hepatic fibrosis.<sup>59–62</sup> No other antidiabetic agent other than rosiglitazone, a TZD, has shown benefit in the treatment of NAFLD/NASH.<sup>111,112</sup>

#### Safety concerns

#### Fat weight gain

Weight gain is common with pioglitazone therapy, typically amounts to  $\sim 2$  to 3 kg of fat mass over 1 year<sup>16,113,114</sup>, and is dose related.113,115 Of note, the greater is the weight gain, the greater is the decline in HbA1c and the greater are the improvements in insulin secretion and insulin sensitivity.94,99,116 How is this explained? Pioglitazone causes an increase in body weight by stimulating PPARy receptors in the hypothalamus to augment appetite.<sup>117</sup> However, pioglitazone simultaneously stimulates PPARy receptors in subcutaneous adipocytes to induce genes involved in adipogenesis.<sup>118</sup> The newly formed, smaller fat cells take up FFA leading to a reduction in the plasma FFA concentration and decreased flux of FFA into liver, muscle and visceral fat depots. In addition, pioglitazone stimulates PPARy coactivator-1 (PGC-1) which is the master switch for mitochondrial biogenesis.<sup>119,120</sup> This causes transcription of mitochondrial genes involved in fatty acid oxidation, resulting in a further reduction in the intramyocellular and hepatocyte lipid content with reversal of lipotoxicity.<sup>2,55,121</sup> It is noteworthy that weight gain, not weight loss, was associated with increased survival in the PROactive study.<sup>122</sup> This observation suggests that pioglitazone also mobilizes fat out of the arterial wall (see preceding discussion).

It is notable that no specific adverse effects of the fat weight gain have been observed in T2DM patients treated with pioglitazone for up to 3-6 years.<sup>16,17,23,62,123</sup> Importantly, the weight gain is dose related and can be minimized by not exceeding a dose of 30 mg/day,<sup>113</sup> the point at which ~80% of the drug's glucose-lowering efficacy is observed. Combination therapy of pioglitazone with metformin minimizes the weight gain,<sup>124</sup> while combination therapy with a SGLT2 inhibitor<sup>19,20</sup> or with a GLP-1 RA<sup>19,125,126</sup> reduces both the weight gain and fluid retention.

#### Fluid retention and HF

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When used as monotherapy, oedema is observed in 5%–10% of pioglitazone-treated individuals and, like weight

gain, is dose related.<sup>113,115</sup> When used in combination with a sulphonylurea or insulin, the incidence of oedema is increased further.<sup>115</sup> The oedema results from two factors: peripheral vasodilation<sup>127</sup> and renal sodium retention.<sup>128</sup> Despite increased total body sodium, blood pressure consistently declines,48,49,56 indicating that the drug's predominant effect is on the vasculature to decreased vascular tone, and that sodium retention is secondary to the vasodilation. Pioglitazone has no apparent negative effect on LV function48,49 and improves diastolic dysfunction.48–52 Nonetheless, pioglitazone should not be used in T2DM patients with symptomatic HF since fluid accumulation in a noncompliant ventricle can precipitate HF in such individuals, leading to clinical deterioration.<sup>115</sup> Salt and water retention respond best to diuretics that act in the distal tubule such as spironolactone, triamterene and amiloride.115 Patients should be instructed to report new oedema or dyspnoea to their physician. If more than trace oedema is present, treatment with one of the distally acting diuretics should be instituted and/or the dose of pioglitazone reduced. Of note, in the IRIS study,<sup>17</sup> the number of patients who developed HF was similar in the pioglitazone-treated (n=74) and placebo-treated (n=71) groups and this study did allow for dose reduction for oedema or weight gain not responding to initial lifestyle recommendations.

#### Bone fractures

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An increase in bone fractures has been reported in T2DM individuals treated with TZDs.<sup>17,129–132</sup> The fractures primarily affect postmenopausal women, occur in the distal long bones of the hands and feet and are related to trauma. One study has reported an increase in fractures in men,<sup>17</sup> while some studies have failed to observe any increase in fractures in either sex.<sup>46</sup> The excess fracture risk amounts to 0.8 fractures per 100 patient-treatment years (1.9 vs 1.1 in pioglitazone vs comparator-treated group).<sup>129–132</sup> Fractures are uncommon in premenopausal women and men. Pioglitazone should be used cautiously or not at all in individuals at high fracture risk, including postmenopausal women with osteoporosis or those with prior fracture.

#### Bladder and cancer

In PROactive,<sup>16</sup> there was a nonsignificant increase in the number (16 vs 6, p=0.069) of patients who developed bladder cancer. Before unblinding of the results, external experts adjudicated that 11 cases could not plausibly be related to treatment (due to the temporal sequence of drug exposure and cancer diagnosis), leaving six cases in the pioglitazone group and three cases in the placebo group (p=0.309). Of note, there were significantly fewer cases of breast cancer (3 vs 11, p=0.034) in the pioglitazone-treated group and the overall incidence of cancer was similar in both groups. Also, after 10 years of follow-up, the

incidence of bladder cancer was similar in pioglitazonetreated versus placebo-treated subjects (28 vs 26, respectively).23 After PROactive, the FDA requested that the manufacturer of pioglitazone initiates a prospective study to examine the relationship between pioglitazone and bladder cancer. A midpoint analysis of this 10-year study<sup>133</sup> involving 193,099 patients revealed no significant association between pioglitazone and bladder cancer (HR=1.2, 95% CI=0.9–1.5, p=NS), but those who were exposed for at least 2 years had a small increased risk (HR=1.4, 95% CI=1.0-2.0). The 10-year follow-up data, however, failed to find any such association between pioglitazone and bladder cancer with sensitivity analyses showing that the neutral effect was present irrespective of dose and duration of therapy (HR = 1.06, 95% CI = 0.89–1.26, p = NS).<sup>134</sup> In a multinational cohort135 involving 1.01 million T2DM patients with greater than 5.9 million person-years, the HR for bladder cancer with pioglitazone and rosiglitazone was 1.01 and 1.00, respectively (both p = NS). In the recently published IRIS study,17 no increase in bladder cancer was observed in the pioglitazone group (0.6% vs 0.4%, p=0.37). Based upon the preceding body of evidence, however, the FDA still cautions about this risk and recommends that pioglitazone not be used in diabetic patients with active bladder cancer or history of bladder cancer.

#### Summary

As we transition to a new evidence-based era of T2DM management in patients with CVD,<sup>15</sup> it is imperative that we choose therapies that not only improve glycaemic control but also improve CV outcomes-the latter representing the greatest cause of mortality in this population. Pioglitazone has been shown to reduce MACE (MI, stroke and CV mortality) in multiple studies including PROactive,<sup>16</sup> IRIS,<sup>17</sup> meta-analyses of multiple prospective studies;<sup>21,22</sup> to reduce CV events and mortality in several large observational studies;24-26,40 to retard the anatomical progression of coronary and carotid atherosclerosis in PERISCOPE,27 Chicago28 and ACT NOW (Table 2).<sup>102</sup> Pioglitazone is the only available insulin-sensitizing agent and has a potent beneficial effect to improve and preserve beta-cell function, leading to a durable reduction in HbA1c (Table 2). Pioglitazone also corrects multiple components of the metabolic syndrome and is an effective treatment for NASH/NAFLD. Side effects remain a concern but can be mitigated by optimizing dosing strategies and combining therapy with other medications (metformin, SGLT2i, GLP-1 RA) that promote weight loss and sodium excretion. The benefit to risk ratio of pioglitazone is very favourable when caution is employed to avoid the known side effects of the drug (Table 2). Moreover, pioglitazone is now generically available and some 50 times less expensive than many branded glucose-lowering drugs with recent CV benefits. It, therefore, represents a highly afford-

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Table 2. Benefits and risk associated with pioglitazone therapy.

Benefit		Ri	Risk	
•	Potent, durable HbA1c reduction	•	Fat weight gain – but decreases visceral, hepatic and muscle fat content	
•	Low risk of hypoglycaemia	•	Fluid retention/heart failure	
•	Improves insulin resistance	•	Bone fractures (distal long bones, trauma related)	
•	Improves beta-cell function	•	Bladder cancer (not established)	
•	Prevents IGT progression to T2DM			
•	Improves cardiovascular risk factors (↑HDL, ↓triglyceride, ↓blood pressure, ↓inflammation)			
•	Reduces microalbuminuria			
•	Decreases cardiovascular events in high-risk diabetic patients (PROactive, IRIS, meta-analysis)			
•	Reduces cardiovascular events in diabetic patients with chronic kidney disease			
•	Improves endothelial dysfunction			

Improves NASH/NAFLD

Improves NASH/NAFLD

IGT: impaired glucose tolerance; T2DM: type 2 diabetes mellitus; HDL: high-density lipoprotein; NASH/NAFLD: nonalcoholic steatohepatitis/nonalcoholic fatty liver disease.

able option for the treatment of patients with T2DM, especially those with prevalent CVD.

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#### **ORCID** iD

Ralph A DeFronzo D https://orcid.org/0000-0003-3839-1724

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**Original Article** 

### Bariatric surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleukin-6

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Safwaan Adam<sup>1,2</sup>, Yifen Liu<sup>1</sup>, Tarza Siahmansur<sup>1</sup>, Jan H Ho<sup>1,2</sup>, Shaishav S Dhage<sup>1,2</sup>, Rahul Yadav<sup>3</sup>, John P New<sup>1,4</sup>, Rachelle Donn<sup>1</sup>, Basil J Ammori<sup>1,5</sup>, Akheel A Syed<sup>1,4</sup>, Rayaz A Malik<sup>1,6</sup>, Handrean Soran<sup>1,2</sup> and Paul N Durrington<sup>1</sup>

#### Abstract

**Background:** Reaven originally described the clustering of insulin resistance/hyperinsulinaemia, obesity (particularly visceral), altered cytokine levels, glucose intolerance, hypertriglyceridaemia and low high-density lipoprotein cholesterol. Subsequently, a potentially highly atherogenic small, dense low-density lipoprotein was also reported. We have studied the effect of bariatric surgery on this and other risk factors for atherosclerosis.

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**Methods:** Forty patients (20 with type 2 diabetes mellitus) undergoing bariatric surgery were studied before and I year after bariatric surgery.

**Results:** Twelve months after bariatric surgery, median body mass index had decreased from 49.5 to  $36.5 \text{ kg/m}^2$ , fasting insulin from 21.3 to 7.8 mU/L and insulin resistance (homeostatic model assessment of insulin resistance) from 5.9 to 1.8 (all p < 0.001). Thirteen out of 20 patients had remission from type 2 diabetes mellitus. Highly sensitive C-reactive protein, interleukin-6, fasting triglycerides (p < 0.001) and small, dense low-density lipoprotein (p < 0.001) decreased, while high-density lipoprotein cholesterol increased (p < 0.001) significantly, irrespective of having type 2 diabetes mellitus and/or being treated with statin therapy before surgery.

**Conclusion:** The association between marked weight loss and change in insulin resistance and hyperinsulinaemia with the change in small, dense low-density lipoprotein and interleukin-6 warrants further investigation. Bariatric surgery provides a model for investigating the mechanisms linking insulin resistance/hyperinsulinaemia to atherosclerosis.

#### Keywords

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Insulin resistance, Reaven's hypothesis, bariatric surgery, obesity, metabolic syndrome, triglycerides

#### Introduction

The advent of reliable plasma insulin assays in the 1960s provided the opportunity for Gerald Reaven et al.<sup>1</sup> to discover the association between the insulin response to carbohydrate feeding and serum triglyceride levels. Higher insulin responses were associated with higher triglyceride levels.<sup>1</sup> Reaven and others went on to report that a constellation of other abnormalities was also associated with an exaggerated insulin response in addition to hypertriglyceridaemia, including type 2 diabetes mellitus (T2DM) or a predisposition to it, high-density lipoprotein cholesterol (HDL-C), non-alcoholic steatohepatitis, hypertension, hyperuricaemia and raised plasminogen activator inhibitor-1 (PAI-1), fibrinogen and highly sensitive

<sup>1</sup>Cardiovascular Research Group, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

 $^2\mathrm{Cardiovascular}$  Trials Unit, Manchester University NHS Foundation Trust, Manchester, UK

<sup>3</sup>Department of Diabetes and Endocrinology, Warrington and Halton Hospitals NHS Foundation Trust, Warrington, UK

<sup>4</sup>Department of Diabetes, Endocrinology and Obesity Medicine, Salford Royal NHS Foundation Trust, Salford, UK

<sup>5</sup>Department of Surgery, Salford Royal NHS Foundation Trust, Salford, UK

<sup>6</sup>Weill-Cornell Medicine-Qatar, Doha, Qatar

#### **Corresponding author:**

Paul N Durrington, Cardiovascular Research Group, Faculty of Biology, Medicine and Health, University of Manchester, Core Technology Facility, 46 Grafton Street, Manchester, M13 9WL, UK. Email: pdurrington@manchester.ac.uk

C-reactive protein (hs-CRP).<sup>2-5</sup> This, he termed 'Syndrome X',<sup>2</sup> although it is now more widely known as the metabolic syndrome, particularly when associated with obesity. Initially, Reaven's hypothesis stated that increased insulin levels were the cause of the hypertriglyceridaemia, because insulin was at that time believed to stimulate hepatic very low-density lipoprotein (VLDL) secretion.<sup>1</sup> However, later experiments with adult hepatocytes maintained in tissue culture showed that the primary effect of insulin on hepatic VLDL secretion was inhibitory.6-8 Thus, the hypertriglyceridaemia was due to insulin resistance, rather than hyperinsulinaemia, as previously proposed by Himsworth9 many years previously. Reaven thus modified his hypothesis and extended it to state that both muscle and the liver must be resistant to the action of insulin, at least in relation to their diminished capacity for the uptake of glucose, and therefore, the increased insulin levels were a response to overcome the insulin resistance. That hepatic insulin resistance could also explain the hypertriglyceridaemia of metabolic syndrome and T2DM, was later confirmed in human studies of VLDL kinetics.<sup>10–12</sup> Throughout his life Reaven continued to argue, however, that it was impossible using available techniques and models to separate the effects of insulin resistance from the effects of too much insulin in humans.<sup>13</sup> It is known that insulin resistance due to inherited insulin receptor defects results in reduced glucose uptake and hyperinsulinaemia, but does not lead to hypertriglyceridaemia or hepatic steatosis.<sup>14</sup> However, after its uptake by functioning receptors, insulin regulates multiple intracellular pathways through several signalling mechanisms.<sup>15</sup> It therefore remains entirely possible that some of these are resistant to insulin, whereas, as Reaven originally postulated, others are over-stimulated by the hyperinsulinaemia which develops to maintain euglycaemia. When the increased delivery of insulin is inadequate to overcome insulin resistance to glucose uptake, T2DM develops, but the insulin levels are much higher than in healthy, nonobese, people without diabetes.<sup>16</sup>

The provision of evidence that hyperinsulinaemia/insulin resistance is causal for hypertriglyceridaemia and the other components of the metabolic syndrome was, until the advent of bariatric surgery, hampered by the lack of a means of dramatically reversing it. Drugs which decrease insulin resistance tend to have multiple other actions and the effect of weight reduction through dietary restriction can only be studied when a large proportion of failures are excluded. Bariatric surgery provides a means of substantially and consistently reversing hyperinsulinaemia/insulin resistance. While the substantial decrease in adiposity may explain this reversal of hyperinsulinaemia/insulin resistance, additional mechanisms such as changes in gut hormone profiles due to intestinal bypass may contribute. We, and others, have previously reported a decrease in elevated levels of inflammatory cytokines and an increase in adiponectin after bariatric surgery.<sup>17–20</sup> These cytokines emanate from adipose tissue and those from visceral adipose tissue, particularly interleukin-6 (IL-6), arrive at the liver via the portal circulation,<sup>21</sup> and are believed to be responsible for hepatic resistance to insulin-mediated glucose uptake.<sup>22</sup>

Quite why the metabolic syndrome is associated with an increased risk of atherosclerotic cardiovascular disease (CVD) has never been fully explained. Increases in lowdensity lipoprotein cholesterol (LDL-C) and in its major protein moiety, apolipoprotein B (ApoB), which are definitely causal, are not a feature of metabolic syndrome.<sup>2,23</sup> Moderate hypertriglyceridaemia, typical of the metabolic syndrome has proved controversial as a cause of CVD,<sup>24</sup> and the role of high-density lipoprotein (HDL) in atherogenesis is currently being re-evaluated.<sup>25</sup> We have previously made a preliminary report of a decrease in small, dense low-density lipoprotein (SD-LDL) following bariatric surgery.<sup>18</sup> SD-LDL is increased in hypertriglyceridaemia 26-28 and is particularly susceptible to atherogenic modifications, such as oxidation and glycation.<sup>29,30</sup> Inflammatory cytokines associated with atherothrombosis may also make a major contribution to CVD in metabolic syndrome.<sup>31</sup> In the present study, we have undertaken a comprehensive assessment of the effect of bariatric surgery on insulin secretion and insulin resistance in relation to the change in SD-LDL and IL-6, an upstream regulator of C-reactive protein (CRP),<sup>4</sup> as there is emerging evidence from Mendelian randomisation studies that it has a longer term association with CVD than hs-CRP.32,33 We have sought to establish the basis for the change in insulin secretion and insulin resistance after bariatric surgery, to further understand the basis of the Reaven hypothesis.

#### Methods

#### Study design and patient recruitment

This study was a prospective observational cohort study. Forty patients (8 men and 32 women) with obesity were recruited from the pre-bariatric surgery clinic at Salford Royal Hospital, a Tier 4 specialist weight management service in the North West of England. They all underwent Roux-en-Y laparoscopic gastric bypass surgery.

Prior ethical approval was sought and granted by the Central Manchester Research and Ethics Committee. All patients were given detailed information about the study and each person provided informed consent before taking part in the study.

#### Patient assessments

Patients were asked to attend a morning appointment (between 09:00 and 10:30 h) having fasted from 22:00 h at baseline and then 6 and 12 months after bariatric surgery.

At each visit, a detailed medical history including medication used was assessed. Each participant underwent measurement of their weight and height and body mass index (BMI) was calculated. Blood pressure was measured after resting in a seated position for 15 min, using an Omron HEM 705-CP semiautomatic oscillometric recorder. Fasting venous blood was collected at each visit. Metabolic syndrome was defined using the current revision from the National Cholesterol Education Programme Adult Treatment Panel III.<sup>23</sup>

Complete remission from type 2 diabetes was determined 12 months post-operatively with glycated haemoglobin (HbA1c) below 6.0% (42 mmol/mol) and no active pharmacological therapy, as per the American Diabetes Association consensus statement.<sup>34</sup>

#### Laboratory procedures and analyses

HbA1c and fasting glucose were assessed in the biochemistry laboratory at Manchester University Hospitals NHS Foundation Trust using routine methods. The remaining samples were processed in the Cardiovascular Research Lab at the University of Manchester. Laboratory procedures and measurements were carried out according to our previously described protocol.<sup>18</sup> Serum and ethylenediaminetetraacetic acid (EDTA)-plasma were isolated by centrifugation at  $2000 \times g$  for 15 min at 4°C within 2 h of blood collection. Aliquots for biochemical analysis were frozen at -80°C.

Total cholesterol was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase method and triglycerides by the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase method. HDL-C was measured using a second-generation homogeneous direct method (Roche Diagnostics, Burgess Hill, UK). LDL-C was estimated using the Friedewald formula.<sup>35</sup> ApoB was measured using immunoturbidimetric assays (ABX Diagnostics, Shefford, UK). All these tests were performed on a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK).

Small, dense low-density lipoprotein apolipoprotein B (SD-LDL ApoB; LDL particles of density > 1.044 g/mL) was isolated from plasma and ultracentrifuged at 100,000 r/min (435,680 × g) for 5 h at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK).<sup>36</sup> ApoB in SD-LDL was then determined using an immunoturbidimetric assay (ABX Diagnostics). SD-LDL is thus expressed in terms of the plasma concentration of its ApoB component.

An in-house, antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique using anti-human CRP antibody, calibrators and controls from Abcam (Cambridge, UK) was used to measure hs-CRP. IL-6 was measured by ELISA using kits from R&D Systems (Abingdon, UK). The upper limit (95th percentile) for IL-6 in plasma was 3.1 pg/mL.<sup>37</sup> Plasma insulin was measured with Mercodia ELISA kits from Diagenics Ltd. (Milton Keynes, UK). Homeostatic model assessment of insulin resistance (HOMA-IR) was used to assess insulin resistance,<sup>38</sup> using the formula

HOMA-IR= 
$$| insulin(mU/L) \times glucose(mmol/L) | /22.5$$

The laboratories participated in the UK National External Quality Assessment Service (UKNEQAS, Birmingham, UK) for quality control of general blood chemistry.

#### Statistical analysis

SPSS for Mac (Version 23.0; IBM SPSS Statistics, IBM Corp., Armonk, NY) and GraphPad Prism (Version 7.00; GraphPad Software, La Jolla, CA, USA) were used for analysis of data. Tests for normality were done using the Shapiro-Wilk test, visualisation of histograms and Q-Q plots. When more than two time points were being compared, one-way ANOVA was used for parametric data and Friedman's two-way analysis of variance by Ranks was used for non-parametric data. Specific post hoc pairwise comparisons were done using the Bonferroni correction in SPSS. The McNemar test was used to compare paired categorical variables. The percentage change in variables was determined as the absolute difference between measurements 12 months after surgery and baseline divided by the baseline value (and multiplied by 100). Correlation analysis was done using Pearson's test for parametric and Spearman's test for non-parametric data. A p-value of <0.05 was considered to be statistically significant.

#### Results

#### Clinical characteristics

The baseline and post-operative clinical measures are given in Table 1. The mean age of participants was 48 years. BMI, waist circumference and systolic blood pressure were reduced significantly (p < 0.05), with no significant change in diastolic blood pressure (p=0.15). Of the 40 patients, 20 had T2DM pre-operatively and remitted completely in 13 out of 20 (65%) patients 12 months after surgery (p < 0.001). There was a significant reduction in the number of participants meeting the diagnostic criteria for the metabolic syndrome (p < 0.001) and there was a trend towards reduction in the use of lipid-lowering drugs (p=0.06) after bariatric surgery.

#### Laboratory measurements

Results of the laboratory measurements are shown in Table 2 and Figure 1. In the entire cohort, there were significant (p < 0.05) reductions in the triglycerides, HDL-C, SD-LDL ApoB, hs-CRP, IL-6, HbA1c, glucose, insulin
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Table T. Chinical parameters before	and alter barrachic surger	ry.		
	Baseline	6 months	12 months	Þ
Age at time of surgery (years)	48 (8)			
Female (%)	32 (80)			
Diabetes presence (%)	50.0		17.5	<0.001
Lipid-lowering drugs (%)	57.5		45.0	0.063
Metabolic syndrome (%)	72.5		22.5	<0.001
Body mass index (kg/m²)	49.5 (45.0–57.0)	36.5 (32.8–44.0)†††	33.0 (30.0–38.8) ****	<0.001
Waist circumference (cm)	37 ( 28– 50)	112 (106–125)†††	l04 (95.0−l15) <sup>§§,***</sup>	<0.001
Systolic blood pressure (mmHg)	131 (120–145)	127 (110–140)	119 (109–134)*	0.025
Diastolic blood pressure (mmHg)	75.0 (13.0)	75.0 (13.0)	70.0 (11.0)	0.150
Excess body mass index loss (%)			65.9 (18.0)	

Table 1. Clinical parameters before and after bariatric surgery.

Clinical parameters at baseline, 6 and 12 months after bariatric surgery. Data represented as mean (SD) or median (IQR). Percentages in categorical variables represent proportion of participants with type 2 diabetes, using lipid-lowering therapy or fulfilling the diagnostic criteria for the metabolic syndrome. Remission of type 2 diabetes determined with a glycated haemoglobin below 42 mmol/mol (6.0%) and no active pharmacological therapy as per the American Diabetes Association consensus statement. Metabolic syndrome defined using the current National Cholesterol Education programme Adult Treatment Panel III.

Bold values shows statistically significant results.

Baseline compared to 6 months: p < 0.05; p < 0.01; p < 0.01; p < 0.01. Baseline compared to 12 months: p < 0.05; p < 0.01; p < 0.01.

and HOMA-IR, 12 months post-operatively with intermediate values at 6 months. Total ApoB levels were significantly reduced only at 12 months and total cholesterol and LDL-C did not change significantly.

Subgroup analysis of the patients with and without diabetes showed similar results, except patients without diabetes had a significant reduction (p=0.01) in LDL-C compared to those with diabetes. The significant improvements in triglycerides, HDL-C, SD-LDL ApoB, hs-CRP, IL-6, HbA1c, glucose, insulin and HOMA-IR were seen both in patients using statins (n=23) and those not on statins (n=17). Those using statins had higher serum total ApoB, reflecting a more severe dyslipidaemia phenotype. Patients not on statin therapy showed a greater reduction in total ApoB, but this was not statistically significant (p=0.12) (Supplementary Table 1).

#### Relationships between fasting insulin, HOMA-IR and other metabolic variables

#### Correlations of values at baseline and I 2 months post-operatively

The relationships at baseline between fasting insulin and BMI are illustrated in Figure 2(a) and with HOMA-IR and BMI in Figure 2(c). The association between post-operative fasting insulin and BMI is shown in Figure 2(b) and for HOMA-IR and BMI in Figure 2(d).

Both pre-operatively and 1 year post-operatively, fasting insulin levels correlated significantly with HDL-C levels (r=-0.37; p=0.02 and r=-0.40; p=0.01, respectively). HOMA-IR measurements showed a significant association

with triglycerides (r=0.34; p=0.03) and HDL-C (r=-0.39; p=0.01) at baseline and at 12 months (triglycerides r=0.36; p=0.02, HDL-C r=-0.42; p=0.007). Triglycerides were not significantly correlated with insulin (r=0.22; p=0.18) at baseline, but were weakly correlated at 12 months post-operatively (r=0.31; p=0.05). Pre-operatively, IL-6 correlated with BMI (r=0.43; p=0.009), but not with insulin levels or HOMA-IR. The relationship with BMI was weaker post-operatively (r=0.31; p=0.07) possibly because of the loss of visceral fat. IL-6 correlated with hs-CRP post-operatively (r=0.42; p=0.007).

### Association of change in hyperinsulinaemia and insulin resistance

The percentage change ( $\Delta$ ) in fasting insulin levels between the pre- and post-operative state was related to  $\Delta$ triglycerides (r=0.36; p=0.03),  $\Delta$ hs-CRP (r=0.42; p=0.01),  $\Delta$ IL-6 (r=0.41; p=0.02) and  $\Delta$ BMI (r=0.43; p=0.007). There were also significant correlations between  $\Delta$ HOMA-IR with  $\Delta$ triglycerides (r=0.33; p=0.04),  $\Delta$ hs-CRP (r=0.37; p=0.02) and  $\Delta$ IL-6 (r=0.37; p=0.03).

#### Discussion

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This study shows that a marked reduction in hyperinsulinaemia/insulin resistance in obese people after bariatric surgery ameliorates not only raised triglycerides, hs-CRP and low HDL-C, features of the Reaven syndrome,<sup>2</sup> but also SD-LDL and IL-6, irrespective of the presence of T2DM or statin therapy.

SD-LDL concentration increases with triglyceride levels.<sup>26</sup> It is a cholesterol-depleted low-density lipoprotein

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	All patients $(n = 40)$	(0			Diabetes $(n = 20)$				Non-diabetes (n =	20)		
	Baseline	6 months	12 months	٩	Baseline	6 months	12 Months	d	Baseline	6 months	12 months	đ
TC (mmol/L)	4.74 (3.88–5.34)	4.56 (3.86–5.63)	4.53 (3.99–5.20)	0.588	4.06 (3.75–5.16)	4.43 (3.86–5.16)	4.23 (3.81–5.03)	0.963	5.00 (4.41–5.86)	5.17 (3.90–5.79)	4.64 (3.38 4.64)	0.359
Triglycerides (mmol/L)	1.68 (1.20–2.25)	I.45 (I.02–I.67)	I.I0%,*** (I.0I–I.35)	<0.001	1.88 (1.27–2.64)	1.49 (1.30–1.70)	l.l6*** (l.01–l.43)	<0.001	1.41 (1.09–2.00)	I.27 (I.01–I.61)	I.05* (0.87–I.35)	0.010
HDL-C (mmol/L)	1.18 (1.03–1.34)	1.18 (1.05–1.51)	1.4055,*** (1.28–1.74)	<0.001	1.11 (1.01–1.39)	1.17 (1.09–1.51)	1.40%,** (1.24–1.73)	0.001	1.23 (1.05–1.32)	1.19 (1.01–1.51)	1.46 <sup>§</sup> ,** (1.30–1.76)	0.002
LDL-C (mmol/L)	2.53 (1.95–3.21)	2.79 (1.89–3.87)	2.38 (1.96–3.22)	0.122	2.03 (1.75-2.62)	2.29 (1.85–3.35)	2.15 (1.78–2.73)	0.086	3.10 (2.36–3.92)	3.00 (2.48–3.88)	2.75** (2.03–3.30)	0.011
Total ApoB (g/L)	0.94 (0.79–1.11)	0.96 (0.76–1.23)	0.86 (0.74–1.06)	0.032	0.86 (0.78–1.00)	0.94 (0.77–1.25)	0.83 (0.69–1.04)	0.120	0.96 (0.78–1.19)	0.96 (0.75–1.23)	0.87 (0.79–1.10)	0.099
SD-LDL ApoB	22.1 (16.6–30.5)	11.5**** (8.90–18.7)	10.2*** (6.8–15.6)	<0.001	21.6 (17.8–28.9)	11.7## (8.44–20.5)	10.2*** (6.87–17.2)	<0.001	22.6 (11.3–30.6)	11.2 (9.13–18.4)	10.1*** (6.75–14.1)	0.001
(mg/dL)												
hs-CRP (mg/L)	6.20 (4.11–10.3)	2.66## (1.10-5.00)	1.07§,*** (0.50–2.92)	<0.001	5.78 (3.85–9.29)	2.6†† (0.7–3.96)	1.21*** (0.65–2.52)	<0.001	6.25 (5.18–11.3)	3.69 (1.25–5.40)	1.0055,*** (0.47–3.06)	<0.001
IL-6 (pg/mL)	2.98 (1.44–5.61)	1.55## (0.38-4.13)	1.26*** (0.17–3.06)	<0.001	3.99 (2.39–7.86)	1.39## (0.78–3.43)	1.26*** (0.41–4.50)	<0.001	2.72 (1.09–3.60)	1.80 (0.06–5.09)	1.01** (0.06–2.87)	0.007
HbAIc (mmol/	45 (41–56)	39## (34-41)	36§,*** (33–40)	<0.001	56 (48–75)	40†† (35–46)	37*** (34-41)	<0.001	42 (38–44)	37 (33–40)	35* (32–37)	<0.001

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<0.001 <0.001

5.08<sup>†</sup> (4.70–5.62)

5.76 (5.14–6.14)

0.004

5.66\*\* (4.67-6.96)

<0.001 6.98 (5.24–12.5)

4.89\*\*\* (4.67-5.82)

5.29# (4.83-6.01)

Glucose (mmol/L) 6.03 (5.24–7.00)

(lom

Table 2. Laboratory values before and after bariatric surgery.

TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; ApoB: apolipoprotein B; SD-LDL ApoB: small, dense low-density lipoprotein apolipoprotein B; hs-CRP: highly sensitive 4.80\*\*\*\* (4.65–5.10) 7.34\*\*\* (5.22–10.8) 1.58\*\*\* (1.13–2.31) 8.58<sup>††</sup> (6.77–13.7) 1.92† (1.52–3.18) 19.9 (15.3–26.6) 5.47 (3.48-6.81) <0.001 <0.001 8.90\*\*\* (5.60-16.4) 2.39\*\*\* (1.22-4.13) 5.97 (4.90–8.59) 9.60† (6.58–16.5) 2.68† (1.80–4.93) 22.9 (13.8–55.9) 8.24 (5.53–13.7) <0.001 <0.001 7.75\*\*\* (5.60–11.7) 1.76\*\*\* (1.22–2.65) 21.3 (14.5–37.8) 9.04## (6.73–13.9) 5.89 (4.17–11.1) 2.56## (1.54–4.34) Insulin (mU/L) HOMA-IR

C-reactive protein; IL-6: interleukin-6; HbA1 c: glycated haemoglobin; HOMA-IR: homeostatic model assessment of insulin resistance.

Laboratory variables at baseline, 6 and 12 months after bariatric surgery in the entire cohort, patients with diabetes and patients without diabetes. Values represented as median (interquartile range)

Bold values shows statistically significant results.

Baseline compared to 6 months: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. Baseline compared to 12 months: \*p < 0.05; ++p < 0.01; ++p < 0.001. 6 months compared to 12 months: \*p < 0.05; +\*p < 0.01; +\*p < 0.01. 6 months compared to 12 months: \*p < 0.05; +\*p < 0.01; +\*p < 0.001.

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**Figure 1.** The responses to bariatric surgery in the whole 40 patients and in those with and without type 2 diabetes separately before and at 6 and 12 months post-operatively in (a) fasting triglycerides, (b) high-density lipoprotein cholesterol (HDL-C), (c) small, dense low-density lipoprotein apolipoprotein B (SD-LDL ApoB), (d) highly sensitive C-reactive protein (hs-CRP), (e) interleukin-6 (IL-6) and (f) insulin resistance (HOMA-IR). Bars are median + 75th percentile. Baseline compared to 6 months: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

Baseline compared to 12 months: p < 0.05; p < 0.01; p < 0.01.

6 months compared to 12 months: p < 0.05; p < 0.01; p < 0.001.

(LDL), the presence of which is not evident from measurement of LDL-C. It contributes to total serum ApoB and is the cause of hyperapobetalipoproteinaemia,<sup>39</sup> but the majority of ApoB-containing lipoproteins are of lower density so that even when total ApoB is measured its presence may not be obvious. It is likely that it is the cause of the association between hypertriglyceridaemia and CVD.<sup>24,26,27</sup> SD-LDL is more susceptible both to oxidative and glycative modification than less dense LDL species.<sup>29,30</sup> Both oxidatively modified and glycated LDL, unlike more buoyant, unmodified LDL, are rapidly taken up via scavenger receptors on macrophages in tissue culture to become foam cells similar to those in atheromatous lesions.<sup>40</sup> In our series of patients, the SD-LDL concentration of 22.1 mg/dL at baseline declined to 10.2 mg/dL 12 months after bariatric surgery, restoring its levels close to the median value in a healthy population (14 mg/dL for men and 9 mg/dL for women).41

A raised level of hs-CRP is recognised as a feature of metabolic syndrome<sup>2</sup> and is closely associated to the risk of developing CVD.<sup>4</sup> However, evidence that hs-CRP is causally related to CVD has not been forthcoming.<sup>4</sup> On the other hand IL-6, a major regulator of hepatic CRP secretion,

unlike CRP, has been found in Mendelian randomisation studies to be linked to atherosclerosis.4,32,33 Many adipokines are released both from peripheral and visceral adipose tissue, which have the potential to contribute to hepatic insulin resistance and secretion of CRP, but those released from visceral fat may have a special place in the genesis of the metabolic syndrome, because they arrive at the liver through the portal vein and may do so at higher concentration than those arriving by the hepatic artery after dilution in the systemic circulation. IL-6 was found in much higher concentration in portal blood than in systemic arterial blood by Fontana et al.<sup>21</sup> in patients undergoing gastric bypass surgery for obesity. Tumour necrosis factor- $\alpha$ , resistin, macrophage chemoattractant protein-1 and adiponectin concentrations were similar in the portal vein and radial artery. Portal vein IL-6 concentration also correlated directly with systemic CRP. The decrease in IL-6 after bariatric surgery, suggests that it could be associated with the decrease in hs-CRP and other features of hepatic insulin resistance. Recently, reduction in IL-6 levels similar to that reported here, but achieved by administration of a monoclonal antibody to interleukin-1β, was reported to be associated with decreased atherosclerotic CVD incidence.31

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**Figure 2.** Fasting insulin and homeostatic model assessment of insulin resistance (HOMA-IR) as a function of body mass index (BMI) at baseline [panels (a) and (c)] and 12 months after bariatric surgery [panels (b) and (d)].

The findings of this and earlier reports in which the effects of the decrease in insulin resistance accompanying weight loss on components of the metabolic syndrome have been investigated,<sup>17-20</sup> provide powerful support for Reaven's hypothesis. Bariatric surgery which induces the most dramatic decreases in hyperinsulinaemia/insulin resistance is a model which could yield an even greater understanding of, for example, the mechanism by which atheroma risk is increased. Our finding of a decrease in SD-LDL should lead to exploration of the effects of weight loss due to bariatric surgery on modified, potentially highly atherogenic LDL subspecies, such as oxidised and glycated LDL. Furthermore, it could lead to some resolution of the conflict which exists between which components of the metabolic (Reaven) syndrome are due to resistance to insulin (too little insulin action) and which are due to hyperinsulinaemia itself (too much insulin action). In recent years, it has often been forgotten that, because insulin regulates several intracellular signalling pathways controlling a variety of processes, while its effects will be deficient in pathways resistant to it, in others the high levels of insulin produced to attempt to maintain euglycaemia may hyperstimulate non-resistant pathways.

An example might be the regulation of sex hormone-binding globulin (SHBG), which is decreased in insulin resistance, leading to increased free androgen levels in both men and women.42,43 This at least partly explains the androgenisation of insulin resistant women and thus their male pattern (visceral; central) obesity and hirsutism. Despite the association of insulin resistance with decreased SHBG, however, tissue culture experiments with human hepatocytes reveal insulin to have an inhibitory action on SHBG production.44,45 Thus, unlike the VLDL production pathway where insulin resistance decreases the inhibitory effect of insulin despite the increase in its concentration in response to resistance to glucose uptake, the pathway for the production of SHBG must escape resistance to the action of insulin and be inhibited by hyperinsulinaemia. The model of bariatric surgery may provide opportunities for futher study of this mechanism as it may for other phenomena associated with the hyperinsulinaemia of insulin resistance, such as the pathways linking metabolic syndrome to hyperuricaemia.46

It has been difficult to distinguish components of the metabolic syndrome due to resistance to insulin action

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and those hyperstimulated by the ensuing increase in insulin secretion to maintain euglycaemia as first discussed in Kim and Reaven.13 We found that the strength of the associations between change in components of the metabolic syndrome, such as triglycerides and HDL-C, and change in fasting insulin and HOMA-IR were similar with perhaps the suggestion that triglyceride concentration was more closely related to insulin resistance and HDL-C to fasting insulin levels. It has been suggested that the insulin clamp technique would be a better means of assessing insulin resistance rather than HOMA-IR, which relies on the ratio between fasting insulin and glucose.<sup>38</sup> However, this argument is less persuasive when it is considered that in insulin clamping, the insulin is administered into the systemic rather than the portal circulation into which it is secreted physiologically.<sup>47</sup> This means that physiologically the liver is subject to much higher levels than peripheral tissues and the insulin clamp is thus measuring insulin resistance to glucose uptake in tissues, such as skeletal muscle, while hepatic uptake is relatively unaffected by insulin arriving by the hepatic artery. Presumably, HOMA-IR, however, represents the contribution of both hepatic and peripheral glucose disposal. At the time of bariatric surgery, direct measurement of hormones and metabolites in portal venous blood can be undertaken,<sup>21</sup> but repetition of this after weight loss is not possible, at least in human models. Nonetheless, discovering which processes are resistant to insulin and which are over-stimulated by the accompanying hyperinsulinaemia could be important therapeutically.

We conclude that bariatric surgery provides an excellent model to dissect Reaven's hypothesis, to discover the mechanisms by which its components, such as raised SD-LDL and IL-6, cause atherosclerosis. It also raises questions about the respective roles of insulin resistance and of hyperinsulinaemia on different components of the metabolic syndrome.

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#### Supplemental material

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#### **ORCID** iD

Safwaan Adam Dhttps://orcid.org/0000-0001-8004-1897

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**Original Article** 

## Impact of race/ethnicity on insulin resistance and hypertriglyceridaemia

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Viraj Raygor<sup>1</sup>, Fahim Abbasi<sup>1</sup>, Laura C Lazzeroni<sup>2,3</sup>, Sun Kim<sup>1,4</sup>, Erik Ingelsson<sup>1,4,5</sup>, Gerald M Reaven<sup>4,5\*</sup> and Joshua W Knowles<sup>1,4,5</sup>

#### Abstract

**Objective:** Insulin sensitivity affects plasma triglyceride concentration and both differ by race/ethnicity. The purpose of this study was to provide a comprehensive assessment of the variation in insulin sensitivity and its relationship to hypertriglyceridaemia between five race/ethnic groups.

**Research design and methods:** In this cross-sectional study, clinical data for 1025 healthy non-Hispanic White, Hispanic White, East Asian, South Asian and African American individuals were analysed. Insulin-mediated glucose disposal (a direct measure of peripheral insulin sensitivity) was measured using the modified insulin suppression test. Statistical analysis was performed using analysis of co-variance.

**Results:** Of the study participants, 63% were non-Hispanic White, 9% were Hispanic White, 11% were East Asian, 11% were South Asian and 6% were African American. Overall, non-Hispanic Whites and African Americans displayed greater insulin sensitivity than East Asians and South Asians. Triglyceride concentration was positively associated with insulin resistance in all groups, including African Americans. Nevertheless, for any given level of insulin sensitivity, African Americans had the lowest triglyceride concentrations.

**Conclusion:** Insulin sensitivity, as assessed by a direct measure of insulin-mediated glucose disposal, and its relationship to triglyceride concentration vary across five race/ethnic groups. Understanding these relationships is crucial for accurate cardiovascular risk stratification and prevention.

#### **Keywords**

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Insulin resistance, race, triglyceride, modified insulin suppression test

#### Introduction

The risk of cardiometabolic conditions including type 2 diabetes, dyslipidemia and atherosclerotic cardiovascular disease varies across race/ethnic groups.<sup>1-4</sup> While the reasons for this variability are not completely understood, it has been postulated that underlying differences in insulin sensitivity<sup>5–8</sup> may be partly responsible. Insulin sensitivity varies widely in apparently healthy individuals.9-13 Although variability in insulin action does not seem to be unique to any given race/ethnic group, the degree of insulin sensitivity does appear to vary with race/ethnicity, at least in small studies mostly utilizing surrogate estimates of insulin sensitivity.9,10,14,15 Decreased insulin sensitivity (insulin resistance) is also associated with increases in plasma triglyceride (TG) concentration, an emerging causal risk factor for atherosclerotic cardiovascular disease,<sup>16,17</sup> and there is evidence that the association between insulin resistance and TG levels may also vary as a function of differences in race/ethnicity.18-20 Plasma TG levels may not only reflect insulin sensitivity but are likely to mediate negative consequences of insulin resistance. Given the importance of insulin resistance and compensatory hyperinsulinemia in the pathogenesis of cardiometabolic diseases, we sought to provide a more comprehensive

<sup>5</sup>Cardiovascular Medicine, Stanford Cardiovascular Institute, Stanford University School of Medicine, Stanford, CA, USA

\*Deceased, 12 February 2018.

#### **Corresponding author:**

Joshua W Knowles, Cardiovascular Medicine, Stanford Cardiovascular Institute, Stanford University School of Medicine, Falk CVRC, Room CV273, MC 5406, 300 Pasteur Drive, Stanford, CA 94305, USA. Email: knowlej@stanford.edu

<sup>&</sup>lt;sup>1</sup>Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

<sup>&</sup>lt;sup>2</sup>Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA, USA

<sup>&</sup>lt;sup>3</sup>Department of Biomedical Data Science, Stanford University School of Medicine, Stanford, CA, USA

<sup>&</sup>lt;sup>4</sup>Diabetes Research Center, Stanford University School of Medicine, Stanford, CA, USA

evaluation of the impact of differences in race/ethnicity on insulin resistance and its associated dyslipidemia. This work differs from the many manuscripts that have addressed this issue in the past for three crucial reasons: (1) data are available from five different race/ethnic groups; (2) insulin resistance was quantified by a direct measurement of insulin-mediated glucose disposal, not a surrogate estimate; and (3) the groups were compared not only on their degree of insulin resistance but also on the relationship between insulin resistance and its closest lipid consequence – changes in plasma TG concentration.

#### **Methods**

#### Study design and patient population

Data for this cross-sectional study were obtained from a database containing clinical information for individuals who have previously participated in research studies at Stanford University (Stanford, CA) between 1991 and 2014. To be included, individuals had to have no anaemia (haemo-globin <10 g/dL) or cardiovascular, kidney or liver disease. Subjects were excluded if they had a history of diabetes as defined as fasting glucose  $\geq$ 126 mg/dL by the American Diabetes Association<sup>21</sup> or were taking medications that could affect carbohydrate metabolism. All individuals were categorized through self-identification by the following race/ethnic groups: non-Hispanic White, Hispanic White, South Asian, East Asian and African American.

#### Measurements

All procedures were performed in the Stanford General Clinical Research Center after fasting for 12 h. Subjects had body weight and height measured for calculation of body mass index (BMI; in kg/m<sup>2</sup>). Plasma glucose was determined by the oxidase method (Analyzer 2; Beckman, Brea, CA). Lipoprotein concentrations were performed in the core laboratory at Stanford by standardized methods approved by the Centers for Disease Control and Prevention. Low-density lipoprotein cholesterol (LDL-C) concentration was calculated (except for 26 subjects whose LDL-C could not be calculated due to a TG concentration >400 mg/dL).

Insulin-mediated glucose disposal was measured using the modified insulin suppression test.<sup>22,23</sup> After an overnight fast, individuals were administered a continuous infusion of octreotide acetate ( $0.27 \,\mu g/m^2/min$ ), insulin ( $32 \,m U/m^2/min$ ) and glucose ( $267 \,m g/m^2/min$ ). Blood was sampled every 30 min until steady-state plasma glucose (SSPG) and steady-state plasma insulin (SSPI) levels were achieved. From 150 to 180 min of the infusion, blood was sampled at 10-min intervals. These final four results were used to determine SSPG and SSPI concentrations for each individual. Because octreotide acetate was used to inhibit endogenous secretion of insulin, each subject had a similar SSPI concentration. Therefore, the SSPG concentration for each individual represented an estimate of the ability of insulin to mediate disposal of infused glucose – that is, higher SSPG concentration reflected greater degree of peripheral insulin resistance.

#### Statistical analysis

Continuous variables were analysed using analysis of covariance (ANCOVA). For racial/ethnic differences in insulin sensitivity, the Bonferroni correction was used for seven post hoc pairwise comparisons (non-Hispanic White vs Hispanic White, South Asian, East Asian and African American groups and African American vs Hispanic White, South Asian and East Asian groups). These specific comparisons were chosen a priori to focus on the groups that were most likely to differ based on prior literature and to decrease the number of tests. Categorical variables were analysed using the chi-squared test. Linear regression was performed to examine the relationship between insulin resistance and TG, LDL-C and high-density lipoprotein cholesterol (HDL-C) levels, again using the Bonferroni correction for the seven pairwise comparisons outlined above when appropriate. TG values were log-transformed to improve normality of distribution. For all statistical analyses, a two-sided p value  $\leq 0.05$  was considered statistically significant. All analyses were done using SPSS (Version 24.0, IBM Corp., Armonk, NY). Results are expressed as means with standard deviations and count frequencies with percentages unless otherwise specified.

#### Results

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#### Baseline characteristics

Table 1 summarizes the baseline characteristics of the study population by race/ethnicity and sex. There were 1025 individuals, of whom n=646 (63%) were non-Hispanic White, n=91 (9%) were Hispanic White, n=118 (11%) were South Asian, n=109 (11%) were East Asian and n=61 (6%) were African American. Women comprised 60% of the total sample population, and there was a similar sex distribution across all race/ethnic groups (p=0.1). Only 11.8% of study participants were being treated with anti-hyperlipidemic agents. Of those, 93% were on statins, 6% on fibrates and 1% on ezetimibe.

#### Insulin resistance by race/ethnicity

The unadjusted SSPG levels for males and females in all five race/ethnic groups are provided in Table 2. After adjustments for age, sex and BMI, non-Hispanic Whites and African Americans had significantly lower SSPG levels (i.e. higher insulin sensitivity) than their South Asian and East Asian counterparts (Table 3).

	Non-Hispanic White	Hispanic White	South Asian	East Asian	African American	þ value
N						
Male	273	35	34	42	23	_
Female	373	56	84	67	38	_
Age (years)						
Male	$51\pm10$	$46\pm10$	47±11	$51\pm9$	$47\pm8$	<0.01
Female	$52\pm10$	$48\pm9$	4  ±	$46 \pm 12$	$48 \pm 9$	<0.01
BMI (kg/m <sup>2</sup> )						
Male	$\textbf{29.9} \pm \textbf{4.4}$	$\textbf{32.8} \pm \textbf{5.6}$	$\textbf{27.0} \pm \textbf{4.3}$	$\textbf{28.1} \pm \textbf{3.7}$	$\textbf{30.8} \pm \textbf{5.0}$	<0.01
Female	$\textbf{29.6} \pm \textbf{5.3}$	$\textbf{32.6} \pm \textbf{7.5}$	$\textbf{28.0} \pm \textbf{3.9}$	$\textbf{28.7} \pm \textbf{5.6}$	$\textbf{33.3} \pm \textbf{7.9}$	<0.01
TC (mg/dL)						
Male	$190 \pm 44$	$185\pm39$	$198 \pm 41$	$201 \pm 48$	$174 \pm 38$	0.13
Female	$199 \pm 36$	$197 \pm 32$	$175 \pm 35$	$202 \pm 42$	$184 \pm 33$	< 0.01
TGª (mg/dL)						
Male	$168 \pm 11$	$158\pm29$	$194\pm30$	$195\pm27$	$112\pm36$	0.03
Female	$126\pm5$	$122\pm14$	4±	$ 5 \pm 2$	$100 \pm 16$	0.12
HDLC (mg/dL)						
Male	$42\pm11$	$39\pm9$	$39\pm9$	4I±I3	$43 \pm 13$	0.37
Female	$53\pm14$	$50\pm15$	$48 \pm 11$	$52\pm14$	$49\pm15$	0.02
LDLC (mg/dL)						
Male	$117 \pm 36$	$115\pm28$	$118\pm28$	$130\pm50$	$109 \pm 34$	0.19
Female	$ 2 \pm 3 $	$122\pm24$	$104 \pm 28$	$122\pm33$	$ 15\pm3 $	< 0.01
FPG (mg/dL)						
Male	$99\pm10$	$103\pm9$	$95\pm12$	$101 \pm 10$	$97\pm8$	0.01
Female	$97\pm10$	96±8	$94\pm9$	$98\pm9$	$96\pm10$	0.11

Table I. Baseline characteristics by race/ethnicity and sex

BMI: body mass index; TC: total cholesterol; TG: triglyceride; HDLC: high-density lipoprotein cholesterol; LDLC: low-density lipoprotein cholesterol; FPG: fasting plasma glucose.

Data are shown as mean  $\pm$  standard deviation. The value of  $p \leq 0.05$  is considered statistically significant.

<sup>a</sup>Statistical analysis was performed on natural log-transformed TG values.

Table 2	. SSPG b	y sex and	race/et	hnicity (	unad	justed)	).
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	Non-Hispanic White	Hispanic White	South Asian	East Asian	African American	þ value
Male	$154\pm4$	$204\pm12^{a,b}$	183±12	$166 \pm 11$	4 ± 4	<0.01
	(146, 163)	(181, 226)	(160, 206)	(145, 187)	(113, 169)	
Female	$150\pm4$	$179 \pm 10^{a}$	167±8	$186 \pm 9^{a}$	176±12	<0.01
	(142, 157)	(160, 199)	(151, 183)	(168, 204)	(152, 199)	

SSPG: steady-state plasma glucose; SE: standard error.

Data shown as mean  $\pm$  SE and 95% confidence interval (in parentheses). SSPG values are given in mg/dL. The value of  $p \le 0.05$  is considered statistically significant.

<sup>a</sup>Statistically significant difference compared to non-Hispanic White individuals.

<sup>b</sup>Statistically significant difference compared to African American individuals.

# Relationship between insulin resistance and TG, LDL-C and HDL-C concentration by race/ ethnicity

Linear regression was used to determine the relationship between insulin resistance and natural log-transformed TG levels along with LDL-C and HDL-C levels after adjustments for age, sex and BMI. For all five groups, there was a significant, positive relationship between SSPG and TG levels on the log scale. Additionally, for a given level of insulin sensitivity, African Americans tended to have the lowest TG levels across all five race/ethnic groups, although these differences were only significant compared to non-Hispanic Whites (Table 4). There was no significant relationship between SSPG and LDL-C levels. While there was a significant, negative relationship between insulin resistance and HDL-C levels for all five groups, there was no race/ethnic difference in HDL-C level at a given level of insulin sensitivity. The statistical relationships between insulin resistance and natural log-transformed TG levels

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Non-Hispanic White	Hispanic White	South Asian	East Asian	African American	þ value
5 ±2 ( 47,  56)	69 ± 7 ( 56,  82)	189±6 <sup>a,b</sup> (177, 201)	188 ± 6 <sup>a,b</sup> (176, 199)	45 ± 8 ( 29,  60)	<0.01

Table 3. SSPG by race/ethnicity (adjusted for age, sex and BMI).

SSPG: steady-state plasma glucose; BMI: body mass index; SE: standard error.

Data shown as mean  $\pm$  SE and 95% confidence interval (in parentheses). SSPG values are given in mg/dL. Covariates in the model are evaluated at the following values: age, 49 years; sex, 1.4 (where sex = '1' if female and '2' if male); and BMI, 29.8 kg/m<sup>2</sup>. The value of  $p \le 0.05$  is considered statistically significant.

<sup>a</sup>Statistically significant difference compared to non-Hispanic White individuals.

<sup>b</sup>Statistically significant difference compared to African American individuals.

Table 4. TG leve	by race/ethnicity	(adjusted for age	e, sex, SSPG and BMI).
		<b>(</b> )	, ,

Non-Hispanic White	Hispanic White	South Asian	East Asian	African American	þ value
148±5	24± 4	3 ± 3	I 57 ± I 3	$107 \pm 17^{a}$	<0.01
( 37,  58)	(97, 152)	(106, 156)	(132, 182)	(74, 140)	

TG: triglyceride; SSPG: steady-state plasma glucose; BMI: body mass index; SE: standard error.

Data shown as mean  $\pm$  SE and 95% confidence interval (in parentheses). TG values are displayed in mg/dL; statistical analysis performed on naturallog transformed TG levels. Covariates in the model are evaluated at the following values: age, 49 years; sex, 1.4 (where sex = '1' if female and '2' if male); SSPG, 161 mg/dL; and BMI, 29.8 kg/m<sup>2</sup>. The value of  $p \leq 0.05$  is considered statistically significant. <sup>a</sup>Statistically significant difference compared to non-Hispanic White individuals.

along with LDL-C and HDL-C levels remained the same after exclusion of patients on anti-hyperlipidemic agents, as demonstrated for TG levels in Supplemental Table 1.

The effect of race/ethnicity on log-transformed TG levels was estimated to be  $TG=e(3.913 + 0.002 \times age - 0.004 \times BMI + 0.003 \times SSPG + 0.244 \times sex [-0.257 if African American] OR [-0.011 if East Asian] OR [-0.097 if Hispanic White] OR [-0.026 if South Asian]).$ 

Numeric values for sex are defined as '1' if female and '2' if male. Non-Hispanic White race is the reference group. As an example, the estimated TG concentration for a 50-year-old African American female with a BMI of 28 kg/m<sup>2</sup> and an SSPG level of 175 mg/dL and her equivalent non-Hispanic White counterpart would be 83 and 107 mg/dL, respectively. Alternatively, a TG concentration of 150 mg/dL in a non-Hispanic White male would be equivalent to 116 mg/dL in an African American male of similar age, BMI and level of insulin resistance.

#### Discussion

In this study, we analysed the differences in insulin resistance and its relationship to hypertriglyceridaemia in 1025 healthy individuals from five race/ethnic groups using an intravenous, direct measure of insulin sensitivity. After adjusting for factors known to affect insulin resistance including BMI, we found that non-Hispanic Whites and African Americans had a similar degree of insulin resistance which was lower than that seen in their South Asian and East Asian counterparts. Additionally, for all five groups, there was a positive relationship between insulin sensitivity and TG levels, although for a given level of insulin resistance, African Americans had lower TG concentrations than other race/ethnic groups.

While previous studies have examined variations in insulin sensitivity by race/ethnicity, they have been limited by the usage of surrogate measures of insulin sensitivity and/or comparison of only two to three race/ethnic groups.<sup>9,14,15</sup> The few studies that have used precise, quantitative measures of insulin-mediated glucose disposal have been carried out in considerably smaller sample sizes (n < 44) than the present<sup>10</sup> because direct measurements are costly, time-intensive and moderately invasive.

This analysis helps to confirm some prior findings. Our results in South Asians are similar to those reported by Raji et al.<sup>24</sup> and Laws et al.<sup>10</sup> In the former study, the authors found that 12 healthy South Asians had reduced glucose disposal rates  $(4.7 \pm 0.4 \text{ vs } 7.5 \pm 0.3 \text{ mg/kg/min},$ p < 0.0001) based on the euglycemic-hyperinsulinemic clamp compared to age- and BMI-matched non-Hispanic Whites. In the latter study, the authors showed that 22 South Asian men and women had 60% higher SSPG levels based on the modified insulin suppression test than an equal number of men and women of European ancestry matched by age and BMI. The magnitude of difference in SSPG levels in this study was higher than ours (which was around 25%); however, their sample size was much smaller (22 vs 118 in ours). These findings are also in broad agreement with studies from Kanaya et al.15 that estimated insulin resistance using the homeostasis model assessment of insulin resistance (HOMA-IR). They reported that South Asian individuals had a significantly higher degree of insulin resistance compared to African American, Hispanic White and non-Hispanic White individuals when adjusted

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for age, sex, BMI, waist circumference, smoking and alcohol use.

For other comparisons, however, our results may help arbitrate between prior results and explain discrepant findings when insulin sensitivity is directly measured versus estimated. In particular, there has been some disagreement in the literature about the degree of insulin sensitivity in African or African American populations. In a small study conducted by Goedecke et al.,<sup>25</sup> 15 Black South African women were noted to have the same degree of peripheral insulin sensitivity as 15 White South African women as measured by the euglycemic-hyperinsulinemic clamp. Pisprasert et al.<sup>13</sup> also showed that African American individuals had similar glucose disposal rates compared to Europeans as measured by the euglycemic-hyperinsulinemic clamp (which is closely correlated to the modified insulin suppression test<sup>26,27</sup>). Nevertheless, some studies have shown that surrogate estimates of insulin resistance may be higher in African Americans compared to their European counterparts. Haffner et al.<sup>14</sup> published results on the difference in insulin sensitivity between non-Hispanic Whites and African Americans as measured by the insulin sensitivity index obtained using a frequently sampled intravenous glucose test. In that study, African American subjects were reported to be more insulin resistant than non-Hispanic White subjects after adjustments for age, sex and BMI. This discrepancy may reflect differences in methodology. In that vein, despite having similar levels of insulin sensitivity by euglycemic-hyperinsulinemic clamp, Pisprasert and colleagues showed that African Americans appeared more insulin resistant when assessed by insulin sensitivity index, HOMA-IR and fasting insulin level.13 Although our statistical power was somewhat limited, we found no difference in insulin sensitivity between African American and non-Hispanic White volunteers which, when taken in context with other available data from reference-based measures, suggest that caution should be used in broadly assessing African American populations as insulin resistant.

Overall, these studies highlight a broader issue of reliability for the use of surrogate measures of insulin resistance. In 490 non-diabetic volunteers, Yeni-Komshian et al.<sup>12</sup> studied the accuracy of several surrogate measures of insulin resistance compared to the modified insulin suppression test as a gold standard. They found that the total integrated insulin response to a 75 g oral glucose challenge (OGTT) was the most closely related to the modified insulin suppression test with a Pearson's correlation coefficient of 0.67, which meant that the total integrated insulin response to 75 g OGTT could only account for ~45% of the variability in true insulin resistance. Other surrogate measures of insulin sensitivity such as fasting insulin, fasting glucose/fasting insulin and HOMA-IR were found to have even lower correlation coefficients (0.61, -0.42 and 0.62, respectively). Consistent with this, Ingelsson and colleagues also found that correlations of various surrogate measures of insulin sensitivity based on fasting measures or OGTT with gold standard intravenous insulin sensitivity analyses are generally below 0.7.<sup>28</sup> These observations clearly demonstrate the limitations of using surrogate measures of insulin sensitivity to study race/ethnic differences in insulin resistance.

For a given level of insulin resistance, we found that African Americans have lower TG levels than non-Hispanic Whites. This is consistent with results of prior population-based studies that have shown that African American individuals tend to have lower TG concentrations than their non-Hispanic White counterparts.<sup>29,30</sup> Sumner and Cowie<sup>18</sup> also found that African Americans with insulin resistance defined by HOMA-IR had lower TG levels than comparable non-Hispanic and Hispanic Whites. Because of lower TG levels, African Americans were less likely to meet criteria for metabolic syndrome than their age-, sexand BMI-matched non-Hispanic and Hispanic White counterparts.<sup>31</sup> In part, this difference in TG levels has been hypothesized to be due to increased lipoprotein lipase activity.<sup>32</sup> Despite these findings, African Americans are known to have higher cardiovascular risk than non-Hispanic Whites,<sup>33</sup> raising the concerns that the criteria commonly used for metabolic syndrome may underestimate cardiovascular risk when using standardized cutoffs without consideration of race/ethnicity. Quantification of the differences in TG concentration between race/ethnicity, as is done in this analysis, can help guide accurate risk estimation for metabolic syndrome and subsequent cardiovascular risk.

There are several limitations to this study. First, we had small sample sizes for a few race/ethnic groups, which limits our power to identify all statistically significant differences between groups. Second, we did not have data on physical activity, alcohol use and waist circumference, which may confound the SSPG and TG differences between race/ethnic groups.

#### Conclusion

Insulin sensitivity and its relationship to TG concentration varies among the five examined race/ethnic groups. Non-Hispanic Whites and African Americans have greater insulin sensitivity, as assessed by a direct measure of insulin-mediated glucose disposal, than other race/ethnic groups. Furthermore, at a given level of insulin resistance, African Americans have lower TG concentrations than non-Hispanic Whites. Nevertheless, there was a significant, positive relationship between TG and insulin resistance showing that TG levels do increase with worsening insulin resistance in African Americans, as with other race/ ethnic groups. Understanding these differences is critical for assessing and mitigating cardiovascular risk, particularly in high-risk race/ethnic groups.

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#### Supplemental material

Supplemental material for this article is available online.

#### **ORCID** iD

Joshua W Knowles D https://orcid.org/0000-0003-1922-7240

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Romana S Mughal<sup>1</sup>, Katherine Bridge<sup>1</sup>, Irma Buza<sup>2</sup>, Rita Slaaby<sup>2</sup>, Jesper Worm<sup>2</sup>, Gro Klitgaard-Povlsen<sup>2</sup>, Henning Hvid<sup>2</sup>, Marianne Schiødt<sup>2</sup>, Richard Cubbon<sup>1</sup>, Nadira Yuldasheva<sup>1</sup>, Anna Skromna<sup>1</sup>, Natallia Makava<sup>1</sup>, Grith Skytte-Olsen<sup>2</sup> and Mark T Kearney<sup>1</sup>

#### Abstract

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Insulin and insulin-like growth factor-1 stimulate specific responses in arteries, which may be disrupted by diet-induced obesity. We examined (1) temporal effects of high-fat diet compared to low-fat diet in mice on insulin receptor, insulin-like growth factor-1 receptor, insulin receptor/insulin-like growth factor-1 receptor hybrid receptor expression and insulin/insulin-like growth factor-1-mediated Akt phosphorylation in aorta; and (2) effects of high-fat diet on insulin and insulin-like growth factor-1-mediated Akt phosphorylation and vascular tone in resistance arteries. Medium-term high-fat diet (5 weeks) decreased insulin-like growth factor-1 receptor expression and increased hybrid expression (~30%) only. After long-term (16 weeks) high-fat diet, insulin receptor expression was reduced by ~30%, insulin-like growth factor-1 receptor expression decreased a further ~40% and hybrid expression increased a further ~60%. Independent correlates of hybrid receptor expression were high-fat diet, duration of high-fat diet and plasma insulin-like growth factor-1 (all p < 0.05). In aorta, insulin was a more potent activator of Akt than insulin-like growth factor-1, whereas in resistance arteries, insulin-like growth factor-1 was more potent than insulin. High-fat diet blunted insulin-mediated vasorelaxation (p < 0.01) but had no effect on insulin-like growth factor-1 on vascular tone and Akt activation may have implications in treating obesity-related vascular disease.

#### **Keywords**

Obesity, IGF-I receptors, hybrid receptors

#### Introduction

Acting via their cognate receptors insulin and insulin-like growth factor-1 (IGF-1) respond to environmental cues and nutrient availability to coordinate metabolism and growth.<sup>1</sup> To do this, insulin and IGF-1 may act on multiple tissues, including the vascular endothelium where they activate endothelial nitric oxide synthase (eNOS) activation of the upstream kinase Akt.<sup>2</sup> In aorta, we have shown that insulin and IGF-1 stimulated vasorelaxation and activation of eNOS is blunted in obesity.<sup>2</sup>

The insulin receptor (IR) and IGF-1 receptor (IGF-1R) are heterodimers consisting of two extracellular  $\alpha$ -subunits and two transmembrane spanning  $\beta$ -subunits held together by disulphide bonds.<sup>3</sup> Homology between IR and IGF-1R

is high and as a result they can heterodimerise to form hybrid receptors composed of one IGF-1R $\alpha\beta$  complex and one IR $\alpha\beta$  subunit complex.<sup>4</sup> The proportion of hybrid dimerisation is thought to be a function of the molar fraction of each receptor in the ER.<sup>5</sup> According to this model,

**Corresponding author:** 

Mark T Kearney, Division of Cardiovascular and Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine (LICAMM), School of Medicine, University of Leeds, Leeds LS2 9JT, UK. Email: m.t.kearney@leeds.ac.uk

<sup>&</sup>lt;sup>1</sup>Division of Cardiovascular and Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine (LICAMM), School of Medicine, University of Leeds, Leeds, UK <sup>2</sup>Global Research, Novo Nordisk A/S, Malov, Denmark

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Peptide/protein target	Name of antibody	Manufacturer catalogue #	Species raised, monoclonal or polyclonal	Dilution used
IR-β	Insulin receptor beta 4b8	Cell Signaling, 3025	Rabbit, monoclonal	1:1000
IGF-IRβ	IGF-1 receptor beta, d23h3	Cell Signaling, 9750	Rabbit, monoclonal	1:1000
β-actin	β-actin (13E5)	Cell Signaling, 4970	Rabbit, monoclonal	1:20 000
Phospho-Akt (Ser473)	Phospho-Akt (Ser473) (D9E) XP	Cell Signaling, 4060	Rabbit, monoclonal	1:2000
Akt	Akt (pan)(11E7)	Cell Signaling, 4685	Rabbit, monoclonal	1:1000

#### Table I. Antibody details.

a marked increase in IR leads hybrids to form in preference to IGF-1R homodimers.<sup>6</sup> Hybrid receptors are thought to have a binding affinity similar to the IGF-1R, that is, binding IGF-1, but not insulin, with high affinity.<sup>7</sup> By reducing IR availability, the formation of hybrid receptors has been suggested to have a negative regulatory effect on insulin signalling.<sup>8,9</sup>

In cross-sectional studies in humans with insulin resistance of relatively short duration, increased hybrid receptor expression is not seen,<sup>10</sup> whereas patients with type 2 diabetes have down-regulation of IR and increased expression of hybrids.11 The temporal relationship between expression of IR, IGF-1R and hybrids in obesity and their pathological correlates in the vasculature in vivo remains unclear. Moreover, whether IGF-1 has vasorelaxant effects in resistance arteries and the effect of obesity on these responses is also unclear. To answer these questions we examined (1) the temporal changes in expression of IGF-1R, IR and hybrids in aorta and their correlates in high-fat diet-induced obesity; (2) the effect of different pathological insults associated with obesity on IGF-1R, IR and hybrid expression in human endothelial cells; and (3) the effect of IGF-1 and insulin on resistance vessel tone and Akt phosphorylation and the influence of obesity on these responses.

#### Methods

#### Animals and animal procedures

C57BL/6J male mice were purchased from Jackson Laboratories and acclimatised for 7 days before starting experimental procedures. Mice were maintained in a temperature and humidity-controlled environment on a 12-h light:dark cycle. Male mice were studied in all experiments which were conducted in accordance with accepted standards of humane animal care under UK Home Office Project licence No. P144DD0D6.

#### Diet-induced obesity

Mice were rendered obese by placing them on a 60% highfat diet (HF; diet D12492, Research Diets Inc. New Brunswick, NJ, USA). Age-matched littermate controls were placed on a 10% low-fat diet (LF; diet D12450B, Research Diets Inc. New Brunswick, NJ, USA). All mice were fed standard chow [Special Diet Services, CRM P(PB), Dietex International] until reaching 6 weeks of age, at which point diets were switched to either HF or LF for 2, 5 or 16 weeks.

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#### In vivo examination of glucose homeostasis

In vivo metabolic testing was performed as previously described;<sup>12,13</sup> for glucose tolerance tests (GTT), mice were fasted for 6h, followed by intraperitoneal (IP) injection of 2 mg/kg glucose after which blood glucose was determined at 30-min intervals by tail vein sampling using a portable glucometer (Accu-chek Aviva; Roche Diagnostics, Burgess Hill, UK). For analyses of plasma insulin and IGF-1, blood was sampled at euthanasia from the inferior vena cava. Plasma insulin and IGF-1 were measured using ultrasensitive mouse enzyme-linked immunosorbent assay (ELISA) kits (CrystalChem, Downers Grove, IL and R&D Systems, Bio-Techne, MN) as previously described.<sup>12</sup>

#### Quantification of IRs and IGF-IRs

Mice were euthanised at 2, 5 or 16 weeks after feeding and aortae harvested and snap-frozen. Tissue was processed for analysis by Western blotting to examine changes in receptor protein expression. Samples were mechanically lysed in cell extraction buffer (Invitrogen, Carlsbad, CA, USA) with inhibitors, using a TissueLyser (QIAGEN, Dusseldorf, Germany). Protein was quantified by the bicinchoninic acid assay (BCA) (Sigma-Aldrich, St. Louis, MO, USA). Twenty micrograms of protein was resolved on a 4%-12% Bis-Tris gel (Bio-Rad, Hertfordshire, UK) and transferred to nitrocellulose membranes. Membranes were probed with antibodies diluted in 5% bovine serum albumin (BSA); 1:1000 insulin receptorbeta (clone 4b8), 1:1000 IGF-1 receptor-beta (clone D23H3) and 1:20,000 beta actin (Cell Signaling, MA, USA), before incubation with appropriate secondary horseradish peroxidase (HRP)-conjugated antibody (Dako, Glostrup, Denmark). All antibodies are summarised in

Table 1. Blots were visualised with Immobilon Western Chemiluminescence HRP Substrate (Merck Millipore, Hertfordshire, UK) and imaged with Syngene chemiluminescence imaging system (SynGene, Cambridge, UK).

#### Quantification of hybrid receptors

Hybrid receptor expression was studied by immunoprecipitation and Western blot analysis. Immunoprecipitation: total protein was combined with 30 µL of protein G agarose beads (Roche Diagnostic, Switzerland), 300 µL buffer (100 mM HEPES, pH 7.8, 100mM NaCl, 10mM MgSO4, 0.02% Tween-20) and 1:100 dilution of IGF-1 receptor antibody (D23H3). Ag-Ab immune complexes were allowed to form over 3h at 4°C, after which they were collected using brief centrifugation. Precipitates were washed gently three times in phosphate-buffered saline (PBS)-0.02% Tween-20 before elution with sodium dodecyl sulphate (SDS) buffer. Western blotting: Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were probed with IR- $\beta$  antibody; 1:1000 (4b8), followed by appropriate secondary HRP-conjugated antibody to visualise IR/IGF-1R hybrids. Membranes were re-probed with IGF-1R-B antibody to allow hybrid receptors to be reported as relative levels compared with total IGF-1R protein.

#### IR and IGF-IR gene expression

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Quantitative real-time polymerase chain reaction (PCR) was used to measure mRNA levels of IR and IGF-1Rs. mRNA from aorta was isolated and purified using the RNeasy mini kit (QIAGEN, Dusseldorf, Germany) as per the manufacturer's protocol. Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hertfordshire, UK). Quantitative PCR was then used to determine IR and IGF-1R mRNA expression using specific TaqMan assays (Invitrogen, IR; Mm01211875\_m1, IGF-1R; Mm00802831\_m1). Receptor expression was calculated relative to the average of two housekeeping genes – TATA box–binding protein (TBP; Mm01277042\_m1) and CyclinB (Mm03053893\_gH) – using the formula  $2^{-\Delta Ct}$ .

### Insulin and IGF-1 stimulated Akt phosphorylation in vivo

Mice were injected subcutaneously with either vehicle, native human insulin (Novo Nordisk, Malov, Denmark) or recombinant human IGF-1 (Ipsen, Slough, UK). Dosage was calculated based on the average weight of all lean mice (assuming blood volume does not significantly alter in obese mice). Plasma levels of human IGF-1 and human insulin in the mice were measured using ELISAs (*insulin*; Novo Nordisk, Malov, Denmark. *IGF-1*; Immunodiagnostic Systems, Tyne & Wear, UK) as described previously,<sup>14</sup> in order to confirm equivalent dosing levels between HF and LF mice. After 15 min stimulation, mice were euthanised and the aorta rapidly harvested and snap-frozen. Twenty micrograms of protein was processed for Western blotting. Nitrocellulose membranes were probed with antibodies diluted in 5% BSA; 1:1000 Akt, 1:2000 phosphorylated Akt (Ser473) and 1:20,000 beta actin (Cell Signaling).

#### In vitro assessment of receptor and hybrid expression in human umbilical vein endothelial cells

Cryopreserved human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Stourbridge, UK) and maintained in culture in endothelial cell growth medium at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. At ~70% confluency, cells were treated with the following: 100 nM human recombinant insulin (Sigma-Aldrich, Dorset, UK), 100 nM human recombinant IGF-1 (GroPep, Adelaide, Australia), 10 ng/mL TNF- $\alpha$  (PeproTech, London, UK) 1 µM angiotensin II (Sigma-Aldrich, Dorset, UK), 50 µM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Sigma-Aldrich, Dorset, UK), 25 mM glucose and/or 10 nM insulin (Sigma-Aldrich, Dorset, UK) for 24 h in low-serum (0.5%) medium. Whole-cell lysates were prepared in cell extraction buffer and samples processed for Western blot analysis of IR, IGF-1R and hybrid receptors.

### Resistance vessel vasomotor function in response to insulin and IGF-1

Two-millimetre segments of first-order mesenteric arteries were harvested from LT HF and LF mice and mounted in a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark) containing physiological buffer (mM): KCl 7.4, NaCl 118, NaHCO<sub>3</sub> 15, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, glucose 11, CaCl\_2 2.5, EDTA 0.023 at 37°C, 5% CO\_2 and 95% O\_2. Vessels were equilibrated at a resting lumen diameter of 0.9×L100 (L100 represents vessel diameter under passive transmural pressure of 100 mmHg) in buffer for 30 min. Three potassium-induced constrictions were performed using high potassium buffer and vessels constricting less than 1 mN were excluded from the study. Vessels were pre-constricted with phenylephrine, at a dose yielding approximately 40% constriction obtained with high potassium buffer, and left to stabilise for 10 min. Relaxation to cumulative addition of either insulin (0.001 nM/1 pM to 1 µM) or IGF-1 (0.001 nM to 10 nM) was assessed in preconstricted vessels. A time-matched control recording was also performed following the same protocol, without the addition of insulin or IGF-1. The contractile force of a vessel segment was recorded using PowerLab 4/25-LabChart7 acquisition system (ADInstruments, Oxford, UK).

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	2 weeks		5 weeks		l 6 weeks	
	LF	HF	LF	HF	LF	HF
Weight (g)	26.0 ± 0.4	$\textbf{29.2}\pm\textbf{0.6}^{a}$	$\textbf{25.4} \pm \textbf{0.5}$	$30.8\pm0.5^{\rm a}$	29.8 ± 0.4	$50.6\pm0.6^{\rm a}$
Fasting glucose (mmol/L)	$7.7\pm0.3$	$9.7\pm0.3^{b}$	$\textbf{9.3}\pm\textbf{0.3}$	$13.1 \pm 0.3^{a}$	$8.6\pm0.2$	$12.7\pm0.5^{a}$
GTT-AUC (mmol/L×time)	$19.8 \pm 1.1$	$38.3 \pm \mathbf{2.0^a}$	$11.2 \pm 1.2$	$23.6\pm1.5^{\rm a}$	$10.1\pm0.8$	$46.7\pm3.1^{\rm a}$
Plasma insulin (ng/mL) Plasma IGF-1 (ng/mL)	$\begin{array}{c}\textbf{0.7}\pm\textbf{0.2}\\\textbf{273}\pm\textbf{21.9}\end{array}$	$1.8 \pm 0.5^{\circ}$ 281.9 $\pm$ 33.6	$\begin{array}{c} \textbf{0.6} \pm \textbf{0.1} \\ \textbf{230} \pm \textbf{7.7} \end{array}$	$1.8 \pm 0.2^{a}$ 277 $\pm 10.6^{b}$	$0.8 \pm 0.06$ 219 ± 14	$\begin{array}{c} {\rm 5.9 \pm 0.4^{a}} \\ {\rm 408 \pm 21.6^{a}} \end{array}$

Table 2. Metabolic effects of high-fat (HF) calorie diet for 2, 5 and 16 weeks.

'GTT-AUC denotes GTT-Area Under Curve LF' denotes lean diet, n = 10-25 in each group.

Data are reported as means  $\pm$  SEM.

<sup>a</sup>b < 0.0001.

 $b_p < 0.001$ .

 $^{c}p < 0.05$  compared to the respective control (LF) group.

#### Ex vivo analysis of insulin and IGF- 1 induced Akt phosphorylation in resistance arteries

First-order mesenteric artery segments of 5 mm length were placed into Krebs Ringer solution and stimulated with insulin or IGF-1 at different concentrations (0.001 nM to 1  $\mu$ M) for 15 min at 37°C. Stimulated vessels were snap-frozen, then lysed and sonicated. Samples were analysed by SDS-PAGE and Western blotting.

#### Statistical methods

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Data were analysed using GraphPad Prism software (version 7). For animal studies, one-way analysis of variance (ANOVA) was used to compare the mean value across groups, followed by Tukey's multiple comparisons test. Where differences between two groups were analysed, an unpaired t-test was used with Welch's correction. To study differences in vitro, a paired two-way t-test was utilised.

The results are given as mean  $\pm$  standard error of the mean (SEM). In this study, differences with a *p* value of <0.05 were considered statistically significant. *Multivariate and univariate analysis*: Uni- and multivariate linear regression analysis was performed using SPSS version 21 (IBM Corporation, Armonk, NY) to determine the association between receptor abundance and selected covariates. Standardised regression (beta) coefficients are presented, with \* denoting statistical significance at *p* < 0.05.

#### Results

## Progressive decline in insulin and IGF-1 sensitivity in obesity

We fed mice a HF, obesogenic diet for 2, 5 or 16 weeks; this led to progressive metabolic impairment in comparison to LF fed controls (summarised in Table 2).

## IGF-IR, IR and IGF-IR/IR hybrid receptor expression in aorta during obesity

We studied changes in IR, IGF-1R and hybrid receptor expression in aortic lysates from mice after 2, 5 and 16 weeks of HF and LF. In tissue samples, IR was observed as a double band migrating at 80–100 kDa. We observed IR as a single or double band in tissue but not cell lysates. We suggest this is due to the varying degrees of IR glycosylation in different cell types and tissues, resulting in two migrating populations of IR. We did not observe any discernible difference in the two populations of IR when comparing HF and LF. The level of hybrid receptors was studied by immunoprecipitating IGF-1R and detecting IR in the hybrid receptor by Western blot. The relative level of IR compared to total IGF-1R was determined. The effect of an obesogenic diet was studied over time. After 2 weeks feeding, IR, IGF-1R and hybrid receptor protein expression was unchanged (Figure 1). After 5 weeks of HF, IR expression in aorta was unchanged (Figure 1(a)), whereas IGF-1R expression had declined by 30% (Figure 1(b)) and hybrid receptor expression increased by 38% (Figure 1(c)). After 16 weeks of HF, IR expression had declined by 24% (Figure 1(a)), IGF-1R expression had declined further by 34% (Figure 1(b)) and hybrid receptor expression increased by 62% (Figure 1(c)).

To determine whether the reduction in receptor expression was due to transcriptional changes, real-time PCR was performed on RNA isolated from aorta of mice after 16 weeks of feeding. No changes were observed in IR (Figure 1(d)) or IGF-1R (Figure 1(e)) relative mRNA expression between HF and LF fed mice.

Univariate correlates of hybrid receptor expression were plasma insulin, plasma IGF-1, fasting glucose, body weight, dietary fat content and duration of diet, all p < 0.05. In multivariate analysis, independent predictors of hybrid expression were dietary fat content, duration of diet ingestion and plasma IGF-1, all p < 0.05 (Table 3).



**Figure 1.** Temporal effects of obesity on IR, IGF-IR and hybrid receptor expression in aorta of low-fat (LF) and high-fat (HF) diet fed mice. Data show changes in (a) IR, (b) IGF-IR and (c) hybrid receptor protein at 2, 5 and 16 weeks of feeding. Representative Western blot images are shown with densitometry (a, b and c, n = 10-25 in each group). Relative (d) IR and (e) IGF-IR mRNA is shown in LF and HF mouse aortae at 16 weeks feeding (n=6 in each group). Analysis was performed between gels, and samples were normalised to a single control whole cell lysate which was loaded on all gels. All data are given as mean values  $\pm$  SEM. \*\*p < 0.001, \*\*\*\*p < 0.001

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 Table 3. Independent predictors of hybrid receptor (HR), insulin receptor (IR) and IGF-1 receptor (IGF-1R) expression in mouse aorta.

Covariate	Univariate o	correlation with		Multivariate	correlation with	a
	HR	IR	IGF-1R	HR	IR	IGF-1R
Dietary fat (%)	0.502*	-0.119	-0.161	0.325*	-0.002	-0.007
Diet duration (weeks)	0.348*	-0.386*	-0.556*	0.304*	-0.411*	-0.581*
Body mass (g)	0.585*	-0.168	-0.315*	-0.231	0.185	0.225
Capillary glucose (mmol/L)	0.319*	-0.538*	-0.573*	-0.035	-0.654*	-0.61*
Plasma insulin	0.572*	-0.137	-0.242*	0.282	-0.18	-0.136
Plasma IGF-1	0.536*	0.17	-0.024	0.276*	0.516*	0.3*

IGF-1: insulin-like growth factor-1.

 $\boldsymbol{\beta}$  coefficients presented.

<sup>a</sup>Presented multivariate correlation coefficients account for all five other listed covariates.

\*p<0.05.

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# IGF-IR, IR and IGF-IR/IR hybrid expression in vitro in response to different components of the obesity phenotype

We cultured HUVECs in conditions aiming to recapitulate different components of the obesity phenotype including elevated: insulin, IGF-1, glucose with and without insulin, angiotensin II, hydrogen peroxide and TNF- $\alpha$  for 24 h. Only insulin and IGF-1 reduced expression of their respective receptors despite this hybrid receptor expression remained unchanged (Figure 2).

### Temporal effects of obesity on IGF-1 and insulin stimulated Akt phosphorylation

To examine the temporal effect of obesity on insulin and IGF-1-mediated phosphorylation of the key signalling kinase Akt, we performed in vivo administration of either insulin or IGF-1 to HF and LF fed mice after 2, 5 and 16 weeks.

To determine the optimum dose of IGF-1, we first performed a study in lean mice to examine the effect of equimolar and equipotent (as determined by blood glucose lowering ability) doses of IGF-1 and insulin on aortic Akt phosphorylation. When equimolar concentrations of insulin (4.5 nmol/kg) or IGF-1 (4.5 nmol/kg) were administered, insulin led to a greater decrement in blood glucose and greater increment in phosphorylation of Akt in aorta than IGF-1 (Figure 3(a) and (b)). An IGF-1 dose of 90 nmol/kg stimulated similar blood glucose lowering and Akt phosphorylation as 4.5 nmol/kg insulin. Therefore, in subsequent studies, we used equipotent doses; insulin at 4.5 nmol/kg and IGF-1 at 90 nmol/kg. To ensure that plasma exposure levels would be comparable between LF and HF mice, doses for all mice were calculated based on the average body weight of the LF mice. Plasma exposure levels of human insulin and IGF-1 was assessed with specific ELISAs for insulin and

IGF-1 and we found comparable levels between the LF and HF groups.

After 2 weeks HF, despite no change in receptor expression, both insulin and IGF-1-mediated Akt phosphorylation were blunted (Figure 3(e)). After 5 weeks, HF both insulin and IGF-1-mediated Akt phosphorylation were blunted (Figure 3(f)). By 16 weeks, however, while insulin-mediated Akt phosphorylation remained blunted, IGF-1 mediated Akt phosphorylation was similar in LF and HF fed mice (Figure 3(g)), possibly reflecting an increase in hybrid receptor expression.

#### Resistance vessel relaxation and Akt phosphorylation in response to insulin and IGF-1

We previously demonstrated that 8 weeks HF led to blunting of both insulin and IGF-1-mediated vasorelaxation of the aorta;<sup>2</sup> however, this study did not examine the effect of obesity on resistance vessel function. Here, we show that both insulin and IGF-1 led to vasorelaxation of firstorder mesenteric arteries (Figure 4(a) to (c)). IGF-1, however, was more potent than insulin (Figure 4(d) and (e)). HF resulted in blunted insulin-mediated vasorelaxation (Figure 4(f)) but IGF-1-mediated responses were unaffected (Figure 4(g)). A dose-dependent increase in phosphorylation of Akt was observed with increasing concentrations of insulin and IGF-1 (Figure 4(h) and (i)); however, IGF-1 treatment led to a greater maximal response (Figure 4(j) and (k)). HF-blunted insulin-mediated Akt phosphorylation in first-order mesenteric arteries (Figure 4(h)), but IGF-1-mediated Akt phosphorylation was unaffected by HF (Figure 4(i)).

#### Discussion

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This report describes a number of novel findings of relevance to our understanding of obesity, metabolic disease and the insulin/IGF-1 system, including (1) IR/IGF-1R



**Figure 2.** Effects of supplementation of obesity-related modulators on receptor expression in human umbilical vein endothelial cells in vitro. Data show effects of physiological modulators on (a) IR, (b) IGF-1R and (c) hybrid receptor protein expression. Representative Western blot images are shown with densitometry. All data are given as mean values  $\pm$  SEM. a: basal (0.5% low-serum medium); b: insulin (100 nM); c: TNF- $\alpha$  (10 ng/mL); d: angiotensin 2 (1  $\mu$ M); e: H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M); f: glucose (25 mM); g: glucose (25 mM) + insulin (10 nM); h: IGF-1 (100 nM).

\*p<0.05, \*\*p<0.01 versus control (basal) group (n=6 for each).

hybrid protein level in aorta does not appear to be a primary function of mRNA IR and IGF-1R levels; (2) independent in vivo correlates of hybrid expression are plasma IGF-1, HF and duration of HF feeding period; (3) IGF-1 is a more potent activator of Akt and vasorelaxation in resistance vessels than insulin, whereas we have shown previously that the opposite is true in larger conduit vessels; and (4) after 16 weeks of HF diet, IGF-1-mediated responses in resistance vessels and conduit vessels are preserved, whereas insulin-induced responses are blunted.

#### Hybrid receptor expression does not appear to be simply dependent on the molar fraction of each receptor

Energy and nutrient homeostasis in mammals requires tight regulation and integration of multiple systems, which during periods of cellular and whole organism stress, couple nutrient delivery to energy storage, cell growth and tissue repair.<sup>15</sup> Integral to nutrient homeostasis is the insulin/ IGF-1 system, the development and evolution of which

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**Figure 3.** Temporal effects of high-fat (HF) diet-induced obesity on insulin and IGF-1 stimulated Akt phosphorylation compared to lean low-fat (LF) diet fed mice. Reduction in blood glucose is shown in response to insulin (4.5 nmol/kg) and IGF-1 at equimolar (4.5 nmol/kg) and equipotent (90 nmol/kg) doses in lean mice (a). Phosphorylation of Akt in the aorta of lean mice in response to insulin (4.5 nmol/kg) and equipotent and equimolar doses of IGF-1 (b). Data show level of subcutaneously injected human insulin (c) and IGF-1 (d) in plasma of LF and HF diet fed mice. Differences in insulin (4.5 nmol/kg) and IGF-1 (90 nmol/kg) stimulated phosphorylation of Akt in LF and HF mouse aortae are shown at 2 weeks (e), 5 weeks (f) and 16 weeks (g) of feeding. Representative Western blots and densitometry are shown. All data are given as mean values  $\pm$  SEM (n=6-8 for each group). Bars represent comparisons made between HF and lean groups.

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\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 versus lean vehicle group.

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**Figure 4.** Effects of high fat (HF) diet–induced obesity on mesenteric artery function in response to insulin and IGF-1 compared to lean low fat (LF) diet fed mice. (a) Data show a representative recording of dose-dependent insulin-induced vasorelaxation followed by a time-matched control showing stability of pre-constriction over time. Data show (b) insulin-induced and (c) IGF-1-induced relaxation in pre-constricted mesenteric arteries (first order) taken from LF and HF mice after 16 weeks feeding. Differences in vascular sensitivity to insulin and IGF-1 in (d) LF and (e) HF mice are shown and maximal relaxation achieved with (f) insulin and (g) IGF-1. (h) Insulin and (i) IGF-1-mediated phosphorylation of Akt in LF and HF mesenteric arteries is shown with maximal phosphorylation shown in (i) and (k). All data are given as mean values  $\pm$  SEM.

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occurred before the relatively unusual environmental circumstances of caloric excess experienced by 21st-century humans.<sup>16</sup> As a result, the insulin/IGF-1 system is unable to effectively adapt to the challenge posed by chronic calorie excess and gradually deteriorates giving rise to insulin-resistant type 2 diabetes mellitus and its lethal complications, many of which involve the cardiovascular system.<sup>17</sup>

A hallmark of type 2 diabetes is the increased expression of IR/IGF-1R hybrids which are thought to restrict insulin signalling in favour of IGF-1,5 a scenario we,18,19 and others, have demonstrated may be present in the endothelium<sup>20</sup> and vasculature.<sup>21</sup> Understanding how hybrid receptors are regulated and activated in the vasculature is hence of importance to our understanding of obesity-related perturbation of insulin signalling and vascular dysfunction. In this study, and consistent with cross-sectional studies in humans,10 increased hybrid receptor expression was preceded by insulin (and IGF-1) resistance. We also show that expression of hybrid receptors is closely linked to the duration of high-fat diet ingestion and plasma IGF-1 level. In contrast to elegant studies from Federici et al.,<sup>22</sup> we did not demonstrate independent correlations between hybrid receptor expression and blood glucose or insulin concentration,<sup>23</sup> rather IGF-1 concentration more closely correlated with hybrid expression. It is possible that this is due to the presence of obesity rather than the primary hyperinsulinaemia described by Federici et al.23 An additional explanation could be the use of different tissues - muscle samples as studied by Federici et al may show more sensitivity to hybrid formation following perturbations in insulin and glucose, whereas vascular tissue as in this study may be more sensitive to changes in IGF-1 levels. After 16 weeks of HF, we observed no change in receptor mRNA levels yet found that IR and IGF-1R protein decreased in obese mice. Despite this reduction in total receptor level, the relative expression of hybrid receptors increased. This suggests that regulation of IR and IGF-1R occurs at the translational level or it could be speculated that the internalisation/degradation pathways of the receptors are distinct from hybrid receptors and they are more readily influenced by hormone exposure levels.

#### Insulin and IGF-1 in resistance vessel function

We previously showed that obesity leads to resistance to both IGF-1 and insulin-mediated activation of eNOS and relaxation of the aorta.<sup>2</sup> Studies in humans have shown that IGF-1 increases forearm blood flow consistent with an effect on resistance vessels.<sup>24</sup> McCallum et al.<sup>25</sup> showed that IGF-1-mediated vasodilatation of aorta is blunted in hypertensive rats and Hasdai et al.<sup>26</sup> showed that arteriolar vasorelaxation to IGF-1 is attenuated in experimental hypercholesterolaemia. The effect of obesity on insulin and IGF-1-mediated responses in resistance vessels has been unclear. Here, we show that IGF-1 relaxes resistance vessels and is more potent than insulin. We also show the intriguing finding that obesity blunts insulin-mediated resistance vessel relaxation and Akt phosphorylation, while IGF-1-mediated vasorelaxation and Akt phosphorylation remained intact. These findings reveal a potentially important divergence between insulin and IGF-1 responses in resistance vessels with preservation of IGF-1 responses, when we previously showed that obesity leads to IGF-1 resistance in aorta.<sup>2</sup>

#### Study limitations

A number of limitations should be discussed: we used the semi-quantitative approach of expression levels of receptors to estimate receptor numbers so we cannot comment on the exact numerical relationship between IR and IGF-1R in relation to hybrid receptor formation. In resistance vessels, we were unable to quantify receptor expression due to limited amounts of protein available; it would be of interest in the future to examine receptor expression in resistance vessels as obesity progresses.

#### Conclusion

We have provided a number of insights into changes in the IR/IGF-1R/hybrid receptor system as obesity progresses, showing that after long-term obesity, IGF-1-mediated Akt phosphorylation is preserved in aorta and resistance vessels. Moreover, we show that IGF-1 is a more potent vaso-dilator of resistance vessels than insulin, and after 16 weeks of high-fat diet, while insulin-mediated resistance vessel function is blunted, IGF-1 responses are maintained. These data raise the intriguing possibility that using IGF-1 or manipulating hybrid expression may be an approach to treat obesity-related vascular dysfunction, a possibility that warrants future work.

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R.S.M. and K.B. contributed equally to this manuscript.

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#### **ORCID** iD

Romana S Mughal D https://orcid.org/0000-0002-9101-8905

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**Original Article** 

### Baseline fasting plasma insulin levels predict <sup>2019, Vol. 16(2) 171–177</sup> © The Author(s) 2019 risk for major adverse cardiovascular events among patients with diabetes and high-risk vascular disease: Insights from the ACCELERATE trial

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Anirudh Kumar<sup>1</sup>, Divyang R Patel<sup>1</sup>, Kathy E Wolski<sup>1</sup>, A Michael Lincoff<sup>1</sup>, Sangeeta R Kashyap<sup>2</sup>, Giacomo Ruotolo<sup>3</sup>, Ellen McErlean<sup>1</sup>, Govinda Weerakkody<sup>3</sup>, Jeffrey S Riesmeyer<sup>3</sup>, Stephen J Nicholls<sup>4</sup>, Steven E Nissen<sup>1</sup> and Venu Menon<sup>1</sup>

#### Abstract

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Background: Despite optimal treatment, type II diabetes mellitus remains associated with an increased risk for future cardiovascular events. We sought to determine the association between baseline fasting plasma insulin levels and major adverse cardiovascular outcomes in patients with type II diabetes mellitus and high-risk vascular disease enrolled in the ACCELERATE (Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition with Evacetrapib in Patients at a High Risk for Vascular Outcomes) trial.

Methods: We included all patients with type II diabetes mellitus who had a central laboratory measured fasting plasma insulin level drawn at baseline as part of the study protocol. Hazard ratios were generated for the risk of major adverse cardiovascular outcomes (composite of cardiovascular death, non-fatal myocardial infarction, stroke, hospitalization for unstable angina and coronary revascularization) with increasing quartile of baseline fasting plasma insulin level. We then performed a multivariable regression adjusting for significant baseline characteristics.

**Results:** Among 12,092 patients in ACCELERATE, 2042 patients with type II diabetes mellitus had a baseline fasting plasma insulin level drawn. Median follow-up was 28 months. The study population had a mean age of 66.6 years, 79.2% male and 96.2% had established coronary artery disease. During follow-up, major adverse cardiovascular outcomes occurred in 238 patients (11.6%); of these events, 177 were coronary revascularization (8.7%). We observed a statistically significant relationship between rates of revascularization and rising quartile of baseline fasting plasma insulin level which was not noted for the other individual components of major adverse cardiovascular outcomes. Patients with type II diabetes mellitus who underwent revascularization were noted to have significantly higher baseline fasting plasma insulin levels (27.7 vs 21.4 mU/L, p-value = 0.009) although baseline haemoglobin A1c (6.63% vs 6.55%), body mass index (31.5 vs 31.1 kg/m<sup>2</sup>) and medical therapy were otherwise similar to the group not undergoing revascularization. Following multivariable regression adjusting for significant characteristics including exposure to evacetrapib, the log of baseline fasting plasma insulin level was found to be an independent predictor for major adverse cardiovascular outcomes (hazard ratio = 1.36, 95% confidence interval = 1.09-1.69, p-value = 0.007); this was driven by need for future revascularization (hazard ratio = 1.56, 95% confidence interval = 1.21-2.00, p-value = 0.001).

Conclusion: In a contemporary population of patients with type II diabetes mellitus and high-risk vascular disease on optimum medical therapy, baseline hyperinsulinaemia was an independent predictor for major adverse cardiovascular outcomes and need of future coronary revascularization. These results suggest a pathophysiological link between hyperinsulinaemia and progression of atherosclerotic vascular disease among diabetics.

#### **Keywords**

Plasma insulin, revascularization, major adverse cardiovascular events, diabetes mellitus

<sup>1</sup>Heart and Vascular Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

<sup>2</sup>Endocrinology and Metabolism Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

<sup>3</sup>Eli Lilly and Company, Indianapolis, IN, USA

<sup>4</sup>South Australian Health and Medical Research Institute, The University of Adelaide, Adelaide, SA, Australia

#### **Corresponding author:**

Anirudh Kumar, Heart and Vascular Institute, Cleveland Clinic Foundation, Desk J3-5, 9500 Euclid Avenue, Cleveland, OH 44195, USA. Email: kumara9@ccf.org

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#### Introduction

Type II diabetes mellitus (T2DM) is highly prevalent and poses a significant and rising burden on the health care system.<sup>1</sup> Numerous studies have described an association between T2DM and coronary artery disease (CAD).<sup>2</sup> Although several new agents have recently been proven to reduce cardiovascular risk in T2DM,<sup>3–5</sup> the residual risk of developing atherosclerotic cardiovascular events despite optimal treatment remains significantly higher in this population when compared to non-diabetics.<sup>6,7</sup> While a large proportion of the accentuated risk in patients with T2DM is attributable to the presence of traditional risk factors, further identification of modulating novel risk factors is crucial to developing novel therapeutic interventions.

Previous studies have examined the association between hyperinsulinaemia and incidence of CAD in a variety of clinical settings, although primarily in healthy individuals without T2DM or prior history of established CAD.<sup>8–13</sup> A majority of these studies are now of historic importance as subjects were not optimally medically managed with current guideline-recommended therapies. Consequently, the association between baseline fasting insulin levels and residual risk in high-risk patients with T2DM has not been adequately investigated.

The Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition with Evacetrapib in Patients at a High Risk for Vascular Outcomes (ACCE LERATE) trial was a randomized, double-blinded placebo-controlled trial investigating the use of evacetrapib, a cholesteryl ester transfer protein inhibitor, on patients with high-risk vascular disease.<sup>14</sup> We examined the association between baseline fasting plasma insulin levels and major adverse cardiovascular outcomes (MACE) in patients with T2DM and high-risk vascular disease enrolled in the ACCELERATE trial.

#### Methods

The trial design of ACCELERATE has previously been described.<sup>15</sup> Briefly, approximately 12,000 patients with high-risk vascular disease, including those with recent acute coronary syndrome, peripheral arterial disease, cerebrovascular disease and DM with established history of CAD, were randomized in a 1:1 fashion to evacetrapib 130 mg versus placebo. The trial was event-driven with a primary endpoint of MACE which included cardiovascular death, myocardial infarction, cerebrovascular accident, coronary revascularization or hospitalization for unstable angina all of which were adjudicated by a blinded Clinical Endpoints Committee. Due to clinical futility, the trial was terminated prematurely after accrual of 1363 of the planned 1670 primary endpoint events and a median of 26 months study drug exposure. Follow-up was comprehensive and the end of study visit was completed by 98.8% of patients.

Baseline fasting plasma insulin level was collected as part of the study protocol at randomly selected study sites identified at study initiation. We performed a subgroup analysis among those who had a central laboratory measured fasting plasma insulin level collected at baseline and were known to be diabetic. Patients with type 1 diabetes mellitus and insulin-dependent diabetes mellitus were excluded from this analysis. Baseline patient characteristics, medications and laboratory parameters were compiled. Percentages and means ± standard deviations were computed for categorical and continuous variables, respectively. Categorical variables were compared using the chisquare test or Fisher exact tests, when appropriate, while continuous variables were analysed using the two-tailed Student's t test or the Mann–Whitney U test, when appropriate. Kaplan-Meier methods generated survival curves to graphically demonstrate the risk with increasing quartile in baseline fasting plasma insulin. Multivariable Cox's proportional hazard models with stepwise selection identified significant factors associated with each endpoint. Hazard ratios (HRs) with 95% confidence intervals (CI) are reported for the log of fasting plasma insulin after adjustment for other clinical covariates associated with the endpoint. A *p*-value  $\leq 0.05$  was considered statistically significant.

#### Results

A total of 12,092 patients were enrolled in ACCELERATE. As described in Figure 1, 8236 patients had diabetes mellitus with 166 patients excluded due to insulin dependence; of the remaining patients, 2042 patients with T2DM had a baseline fasting plasma insulin level drawn. The overall median follow-up was 28 months. The average age was 66.6 years, 79.2% were male and 96.2% had established CAD. At baseline, 85.2% of patients were taking an aspirin, 95.4% a statin, 79.1% an angiotensin-converting enzyme inhibitor or angiotensin-receptor II antagonist, and 75.3% a  $\beta$ -blocker. Overall, 83.6% of patients were taking an oral hypoglycaemic agent. At study initiation, baseline low-density lipoprotein cholesterol (LDL-C) was 80.6 mg/dL, high-density lipoprotein cholesterol (HDL-C) was 44.6 mg/dL, triglycerides were 150.5 mg/dL and haemoglobin A1c was 6.6%.

During follow-up, MACE occurred in 238 patients (11.6%); among these events, 177 (8.7%) were coronary revascularization. As seen in Figure 2, Kaplan–Meier event curves demonstrated an increase in MACE by quartiles of baseline fasting plasma insulin level which appeared to be predominantly driven by need for revascularization. Baseline characteristics of patients with and without revascularization following randomization are shown in Table 1. Patients with T2DM who underwent revascularization had a 29.4% higher baseline fasting plasma insulin level (27.7 vs 21.4 mU/L, *p*-value=0.009)



Figure 1. Summary of included patients.

and were younger (65.2 vs 66.7 years, *p*-value=0.020), more likely to be current smokers (16.9% vs 12.0%, *p*-value=0.06), have undergone prior percutaneous coronary intervention (83.6% vs 73.5%, *p*-value=0.004) and have a higher baseline LDL-C (83.9 vs 80.3 mg/dL, *p*-value=0.03). The baseline haemoglobin A1c (6.63% vs 6.55%), body mass index (31.5 vs 31.1 kg/m<sup>2</sup>), baseline medical therapy and remainder of characteristics were similar regardless of need for revascularization during follow-up. Table 2 demonstrates that while there was no difference in use of any oral anti-glycaemic medication (81.9% vs 83.8%, *p*=0.529), those who underwent revascularization were significantly less likely to be prescribed a thiazolidinedione (2.3% vs 6.2%, *p*-value=0.032).

Multivariable regression adjusting for baseline haemoglobin A1c, fasting glucose, log of triglycerides, LDL-C, body mass index, age, race, current smoking, prior percutaneous coronary intervention and receipt of evacetrapib noted log of baseline fasting plasma insulin level to be an independent predictor for overall MACE (HR=1.36, 95% CI=1.09–1.69, *p*-value=0.007) and coronary revascularization (HR=1.56, 95% CI=1.21–2.00, *p*-value=0.001).

#### Discussion

The burden of cardiovascular disease among patients with T2DM remains substantial.<sup>16</sup> Although treatment with multiple new agents has proven to improve cardiovascular outcomes, further mechanistic insight into the modulators of risk in this high-risk population remains a priority. This study demonstrates that among a contemporary patient

population with T2DM and known high-risk vascular disease on appropriate guideline-directed medical therapy, baseline fasting insulin level was an independent predictor for MACE mainly mediated through the clinical outcome of need of revascularization. Our results suggest a pathophysiological link between baseline insulin levels and future risk for atherosclerotic vascular disease progression among patients with T2DM.

Although the association between hyperinsulinaemia and cardiovascular disease has been previously investigated in several patient populations with varied results, minimal contemporary data, if any, exist regarding the association between fasting hyperinsulinaemia and progression of atherosclerotic vascular disease in diabetic patients. In a population without diabetes, the Helsinki Policeman study and a sub-group analysis of the Ouebec Cardiovascular study described fasting plasma insulin levels to be an independent predictor of stable angina and acute coronary syndromes among men without pre-existing cardiovascular or cerebrovascular disease or T2DM.8,9 Similarly, Yanase et al.,<sup>17</sup> García et al.<sup>18</sup> and a subgroup analysis of the Trandolapril Cardiac Evaluation (TRACE) study found endogenous insulin levels to be an independent predictor for recurrent cardiovascular events among nondiabetic patients with established CAD.<sup>19</sup> A subgroup analysis of the Atherosclerosis Risk In Communities (ARIC) longitudinal cohort study demonstrated insulin resistance, as measured by the HOMA-IR, to be associated with risk of incident heart failure among patients without a diagnosis of T2DM, prior myocardial infarction or heart failure.<sup>20</sup> Conversely, the Paris Prospective Study, Caerphilly

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	Revascularized N=177	No revascularization N = 1865	p-Value
Baseline characteristics			
Age (years)	$\textbf{65.2} \pm \textbf{7.9}$	$66.7\pm8.7$	0.020*
Male gender (%)	137 (77.4)	1481 (79.4)	0.529
Caucasian (%)	148 (83.6)	1543 (83.2)	0.766
Body mass index	$31.5\pm5.9$	31.I±6.2	0.500
Current smoker (%)	30 (16.9)	225 (12.1)	0.060
Coronary artery disease (%)	171 (96.6)	1794 (96.2)	0.781
Peripheral artery disease (%)	25 (14.1)	237 (12.7)	0.590
Prior acute coronary syndrome (%)	90 (50.8)	935 (50.1)	0.856
Prior percutaneous coronary intervention (%)	143 (83.6)	1319 (73.5)	0.004*
Prior coronary artery bypass grafting (%)	60 (35.1)	646 (36.0)	0.810
Baseline medical therapy			
Statin (%)	170 (96.0)	1778 (95.3)	0.667
Aspirin (%)	157 (88.7)	1583 (84.9)	0.171
Angiotensin-converting enzyme inhibitor or	142 (80.2)	1473 (79.0)	0.697
angiotensin-receptor II antagonist (%)			
Beta-blockers (%)	137 (77.4)	1401 (75.1)	0.501
Calcium-channel blockers (%)	56 (31.6)	538 (28.8)	0.435
Anti-glycaemic medication (%)	145 (81.9)	1562 (83.8)	0.529
Evacetrapib (%)	86 (48.6)	915 (49.1)	0.904
Baseline laboratory parameters			
Insulin (mU/L)	$\textbf{27.7} \pm \textbf{39.5}$	$21.4 \pm 21.0$	0.009*
Haemoglobin AIc (%)	$\textbf{6.63} \pm \textbf{0.92}$	$\textbf{6.55} \pm \textbf{0.96}$	0.166
LDL-C (mg/dL)	$\textbf{83.9} \pm \textbf{26.4}$	$\textbf{80.3} \pm \textbf{25.9}$	0.030*
HDL-C (mg/dL)	$44.2 \pm 11.6$	44.6 $\pm$ 11.2	0.620
Aldosterone (pmol/L)	$133.7 \pm 148.2$	$\textbf{130.8} \pm \textbf{183.1}$	0.590
High-sensitivity C-reactive protein	$\textbf{2.39} \pm \textbf{3.41}$	$\textbf{3.30} \pm \textbf{8.85}$	0.776

LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol. \*p-value < 0.05 was deemed statistically significant.

 Table 2. Use of oral anti-glycaemic medications among patients who underwent revascularization in comparison to those who did not.

Oral anti-glycaemic medication	Revascularized	No revascularization	þ-Value
3,	N=177	N=1865	,
Biguanide (%)	3 (63.8)	1155 (61.9)	0.616
Sulfonylurea (%)	56 (31.6)	536 (28.7)	0.417
Dipeptidyl peptidase-4 inhibitor (%)	32 (18.1)	286 (15.3)	0336
Thiazolidinediones (%)	4 (2.3)	116 (6.2)	0.032*
Alpha-glucosidase inhibitor (%)	5 (2.8)	65 (3.5)	0.644
Combination drug (%)	10 (5.6)	97 (5.2)	0.798
Other (%)	9 (5.1)	95 (5.1)	0.996

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\*p-value < 0.05 was deemed statistically significant.

prospective study, Busselton study and several others have suggested that plasma insulin levels did not predict the risk of atherosclerotic vascular disease among non-diabetics independent of other cardiovascular risk factors.<sup>10–12</sup>

Several mechanisms have been proposed to explain the association of hyperinsulinaemia with atherosclerotic vascular disease. Reaven<sup>21</sup> introduced the concept of syndrome X, later renamed metabolic syndrome, in which resistance of peripheral tissues to insulin-mediated glucose disposal results in a cluster of risk factors including impaired glucose tolerance, elevated triglycerides, decreased HDL cholesterol, elevated blood pressure and central adiposity. This hypothesis remains controversial, and it is unclear whether the role of plasma insulin is causal versus correlative. Our observations in ACCELERATE suggest that hyperinsulinaemia may increase the risk of progression of atherosclerotic vascular disease through alterations in metabolic processes other than that mediated by dyslipidaemia as participants in

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our study were on statin therapy with optimal LDL levels at baseline. Excess insulin itself may directly predispose patients to cardiovascular events mediated by both an inflammatory prothrombotic state and direct effect on the arterial wall.22 Insulin increases the synthesis of plasminogen activator inhibitor-1 which promotes thrombosis and is associated with vascular inflammation.23,24 Plasminogen activator inhibitor-1 can in turn accelerate development of atheroma within vessel walls that are prone to rupture and has been associated with an increased risk of myocardial infarction.<sup>25,26</sup> In addition, plasminogen activator inhibitor-1 can increase proliferation of mural cellular elements and restenosis after percutaneous coronary intervention.<sup>27</sup> Other mechanisms have also been proposed to explain the close relationship between hyperinsulinaemia, endothelial dysfunction and hypertension, including altered cell membrane ion exchange, enhanced sympathetic and renin-angiotensin-aldosterone system activity and suppressed atrial natriuretic peptide activity.28

Although investigation of the cardiovascular safety and clinical efficacy of newer anti-glycaemic medications is now mandated by the Food and Drug Administration,<sup>29</sup> the management of hyperinsulinism and insulin resistance itself in diabetic patients with vascular disease is not well studied. The Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) study was the first major study to investigate the optimal treatment for patients with T2DM and angiographically defined CAD, comparing outcomes associated with an insulin sensitizing strategy versus those with an insulin provision strategy.30 BARI 2D found no significant difference between the two arms in terms of death or cardiovascular events; however, patients randomized to the insulin-sensitizing arm had less weight gain, higher HDL-C levels, decreased plasma insulin levels and changes in biomarker profiles suggestive of restricted fibrinolysis.31

It is possible to treat insulin resistance with pharmacologic interventions that enhance insulin sensitivity (i.e. metformin, thiazolidinediones). As such, there was an initial enthusiasm for this treatment strategy given several studies suggesting a beneficial effect of these agents on cardiovascular outcomes.32-34 However, the ability of such an approach to improve clinical outcomes compared with weight reduction and exercise alone was tempered by data suggesting limited benefit and possible harm associated with the use of thiazolidinedione drugs.35 Notably, our study population had very low rates of thiazolidinedione or alphaglucosidase inhibitor usage. Furthermore, several clinical trials have demonstrated the failure of intensifying glucose control beyond the current recommendations of the American Diabetes Association to show reductions in cardiovascular events.<sup>36</sup> As such, aggressive lifestyle modification focusing on weight reduction, appropriate diet and increased physical activity is currently the primary therapy for the management of metabolic syndrome.37-39 Although our findings do not support routine use of baseline fasting plasma insulin level for risk stratification, further

studies may be warranted regarding its utility in intensifying medical therapy and risk factor modification. In addition, it may be used as a tool for clinical trial design to identify high-risk patients with T2DM who may be more prone to cardiovascular events and thus reduce the number of patients which need to be enrolled.

#### Conclusion

In a contemporary population of patients with T2DM and high-risk vascular disease on optimum medical therapy, baseline fasting plasma insulin levels was an independent predictor for MACE and the need of future coronary revascularization suggesting a pathophysiological link between hyperinsulinaemia and progression of atherosclerotic vascular disease. Future studies investigating the use of fasting plasma insulin levels as a marker for risk stratification to guide use of adjunctive therapies and programmes to promote intensive lifestyle modifications among diabetic patients with high-risk vascular disease are warranted.

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#### **ORCID** iDs

Anirudh Kumar D https://orcid.org/0000-0002-4413-5439 Govinda Weerakkody D https://orcid.org/0000-0003-0595-5554

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### Glycaemic variation is a predictor of all-cause mortality in the Veteran Affairs Diabetes Trial

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Jin J Zhou<sup>1,2</sup>, Juraj Koska<sup>2</sup>, Gideon Bahn<sup>3</sup> and Peter Reaven<sup>2</sup>; for the VADT Investigators

#### Abstract

Diabetes is associated with substantially increased mortality. Classic risk factors explain a portion of the excess of mortality in type 2 diabetes. The aim of this study was to examine whether visit-to-visit variation in fasting glucose and haemoglobin AIc values in the Veteran Affairs Diabetes Trial were associated with all-cause mortality in patients with type 2 diabetes in addition to other comorbidity conditions, hypoglycaemic events and adverse lifestyle behaviours. The Veteran Affairs Diabetes Trial was a randomized trial that enrolled 1791 military veterans who had a suboptimal response to therapy for type 2 diabetes to receive either intensive or standard glucose control. During the Veteran Affairs Diabetes Trial, fasting glucose and haemoglobin AIc were measured quarterly for up to 84 months. Variability measures included coefficient of variation and average real variability. We found that variability measures (coefficient of variation and average real variability) of fasting glucose were predictors of all-cause mortality, even after adjusting for comorbidity index, mean fasting glucose and adverse lifestyle behaviour during the study. Accounting for severe hypoglycaemia did not weaken this association. Our analysis indicates that in the Veteran Affairs Diabetes Trial, longitudinal variation in fasting glucose was associated with all-cause mortality, even when accounting for standard measures of glucose control as well as comorbidity and lifestyle factors.

#### **Keywords**

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Mortality, glycaemic control, long-term glycaemic variability, hypoglycaemia, type 2 diabetes

#### Introduction

Diabetes is associated with substantially increased mortality.<sup>1-4</sup> Classic risk factors, for example, older age and pre-existing cardiovascular disease (CVD), explain a portion of the excess of mortality in type 2 diabetes (T2D).<sup>5-7</sup> Although improving glycaemic control, usually assessed by mean levels of haemoglobin A1c (HbA1c) or fasting glucose, is a key recommended goal for clinicians to enhance diabetes care,<sup>8-10</sup> the relationship of glucose control with mortality appears more complicated. Data from observational studies have shown J-shaped distributions for mortality and glycaemic control, with not only high HbA1c but also low HbA1c associated with mortality risk.<sup>11-13</sup> Moreover, intensive efforts to lower glucose in more advanced T2D patients have failed to reduce, or even increased, mortality.<sup>14-16</sup>

Recently, several studies reported adverse effects of glycaemic variation on macro- and microvascular complications, as well as risk of hypoglycaemia.<sup>17–22</sup> However, studies to examine the relationship of glycaemic instability with mortality in T2D patients have been limited in number and have varied widely in the patient population

studied, the length of follow-up and in the source and nature of the data collected.<sup>23–26</sup> An investigation using primary care medical record data from the United Kingdom among diabetes patients aged 70 years and older reported the association between glycaemic variability, as measured by variability in HbA1c over a 5-year period, and mortality.<sup>11</sup> Among US military veterans with T2D, Prentice et al.<sup>4</sup> used electronic medical records (EMR) to examine the relationship between HbA1c variability and adverse health outcomes including all-cause mortality. Using data from the only glucose lowering trial that has examined this issue, visit-to-visit glycaemic variability

**Corresponding author:** 

Jin J Zhou, Department of Epidemiology and Biostatistics, Mel and Enid Zuckerman College of Public Health, The University of Arizona, 1295 N. Martin Avenue, Tucson, AZ 85724, USA. Email: jzhou@email.arizona.edu

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<sup>&</sup>lt;sup>1</sup>Department of Epidemiology and Biostatistics, Mel and Enid Zuckerman College of Public Health, The University of Arizona, Tucson, AZ, USA

<sup>&</sup>lt;sup>2</sup>Carl T. Hayden Phoenix VA Health Care System, Phoenix, AZ, USA <sup>3</sup>Edward Hines, Jr. VA Hospital, Hines, IL, USA

(HbA1c and blood glucose) was identified as a strong independent predictor of mortality for mildly hyperglycaemic T2D patients randomized to intensive glucose lowering therapy in the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) trial.<sup>18</sup> However, a critical concern in prior studies is whether unmeasured factors in underlying health or behaviour may confound the relationships of glycaemic variation with mortality. Whereas Prentice et al. adjusted for baseline comorbidity, this may be less completely captured in EMR. Moreover, no studies have adequately considered adverse lifestyle behaviours during follow-up.

Therefore, we used data collected during the Veteran Affairs Diabetes Trial (VADT) to examine the association of time-dependent glycaemic variability with mortality. As extensive data collection was possible during the frequent in person visits, the current analysis was able to more fully account for health status and adverse lifestyle behaviours when examining the association of glycaemic variability with all-cause mortality.

#### **Methods**

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The VADT was a randomized trial that enrolled 1791 military veterans (mean age, 60.4 years) who had a suboptimal response to therapy for T2D (HbA1c > 7.5%) to receive either intensive or standard glucose control.<sup>5,27</sup> HbA1c and fasting glucose were measured every 3 months up to a maximum of 84 months. We excluded observations from the first 6 months of the trial to eliminate the effect of rapid reduction (per protocol) in fasting glucose and HbA1c and excluded individuals with two or fewer measurements of fasting glucose or HbA1c. The primary outcome for this analysis was all-cause mortality.

Lifestyle data were extracted from baseline and quarterly follow-up questionnaires: (1) do patients currently smoke cigarettes: if 'Yes' code as 1, if 'No' code as '0'; (2) do patients exercise regularly: if 'Yes' code as '0', if 'No' code as '1'; (3) do patients adhere to diet: if 'Yes' code as '0', if 'No' code as '1'. To generate adverse lifestyle score, we counted the total number of adverse behaviours over the three questions at each visit. In order to study the contribution of lifestyle score to glycaemic variability, we first categorized visit-to-visit glycaemic variability into the lower 50% and upper 50% and used the lifestyle score in a generalized estimation equation model to estimate its relationship with glycaemic variability. We found that a cumulative (worse) lifestyle score was a modest but statistically significant predictor of increased glycaemic variability [odds ratio (confidence interval, CI), 1.021 (1.011, 1.031), p < 0.0001] and all-cause mortality [hazard ratio (HR) (CI), 1.027 (1.010, 1.044), *p*=0.001].

We compared risks of cumulative mean, maximum and most recent fasting glucose or HbA1c values prior to the mortality event with measures of variability for both fasting glucose and HbA1c. Coefficient of variation (CV) and average real variability (ARV) are frequently used and distinct measures of glycaemic variability.4,18,21,28-30 As previously described,<sup>21</sup> we normalized these by means of fasting glucose and HbA1c, respectively. Variables of glycaemic risk were calculated as continuous and time-dependent covariates in Cox proportional hazard models.<sup>17,31</sup> We first examined quintiles of (CV)log-glucose and ARV-glucose (or similar variability measures using HbA1c) to compare the risks of mortality between high versus low variability groups in an age-adjusted model.<sup>21</sup> Risk of continuous glycaemic variation measures were then modelled after adjusting for: Model 1: age only; Model 2: age and baseline covariates reflecting significant baseline differences in characteristics between those who did and did not die during the study (Table 1) including a modified updated Charlson comorbidity index to reflect diabetes-related comorbidity (Supplementary Table 1; similar results were obtained if using standard Charlson comorbidity index;<sup>32,33</sup> Model 3: age, baseline covariates and cumulative mean of fasting glucose or HbA1c to clarify whether variability measures provided risk prediction beyond standard glucose measures; Model 4: Model 3 and a lifestyle score that was treated as a time-dependent covariate. Finally, we considered whether severe hypoglycaemia could be driving the relationship between glycaemic variability and mortality. We first added the variable cumulative severe hypoglycaemia to Model 4 and second, we repeated analysis after removing all patients with severe hypoglycaemia events.

All statistical analyses were performed using R version 3.4.4 (https://www.r-project.org). A two-sided p < 0.05 was considered statistically significant.

#### Results

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A total of 1659 individuals who had at least two measurements of fasting glucose or HbA1c after the first 6 months were included in the analysis, of which 166 died during the study. The mean and median time to allcause death was 48.5 and 48.4 months. There were on average 18.5 visit fasting glucose and HbA1c measures for individuals within the cohort, and a maximum of 26 measures. The cohort was 90% men and had a mean (SD) age of 64.4 (8.6) years. Several baseline risk factors were associated with mortality, including ethnicity [non-Hispanic White (NHW) or not], diabetes duration, prior CVD event, baseline diastolic blood pressure, baseline high-density lipoprotein (HDL) cholesterol, baseline total cholesterol and the updated Charlson comorbidity index (Table 1).

In an age-adjusted model, for fasting glucose and HbA1c, both log(CV) and ARV show significant trends for increasing risk of mortality with higher quintiles of glucose variability (Figure 1). Compared with quintile 1, individuals with fasting glucose variability in the upper quintiles (i.e. quintiles 2, 3, 4 and 5) had significantly higher risk of all-cause mortality. Although there was a

	All-cause mortality		p-value
	No (n=1493)	Yes (n=166)	
Age (years)	59.71 (8.38)	66.30 (8.18)	<0.001
Treatment (n, %)			
Standard	752 (50.4)	81 (48.8)	0.762
Intensive	741 (49.6)	85 (51.2)	
Sex (n, %)			
Male	1445 (96.8)	165 (99.4)	0.1
Female	48 (3.2)	l (0.6)	
NHW (n, %)			
No	580 (38.8)	47 (28.3)	0.01
Yes	913 (61.2)	119 (71.7)	
Smoking status (n, %)			
No	1253 (83.9)	133 (80.1)	0.413
Yes	240 (16.1)	31 (18.9)	
BMI (kg/m <sup>2</sup> )	31.28 (4.44)	31.11 (4.78)	0.637
Diabetes duration (years)	11.41 (7.39)	12.82 (8.42)	0.023
Prior CVD event (n, %)			
No	927 (62.1)	57 (34.3)	<0.001
Yes	566 (37.9)	109 (65.7)	
History of hypertension (n, %)			
No	415 (27.8)	39 (23.5)	0.414
Yes	1075 (72.0)	127 (76.5)	
Missing	3 (0.2)	0	
History of TZD (n, %)			
No	1205 (80.7)	135 (81.3)	0.931
Yes	288 (19.3)	31 (18.7)	
Charlson Comorbidity Index <sup>a</sup>	1.43 (1.78)	2.74 (2.65)	<0.001
Glycated haemoglobin level (%)	9.4 (1.5)	9.4 (1.6)	0.977
DBP (mmHg)	76 (10)	72.7 (11)	<0.001
SBP (mmHg)	131 (16)	133 (18)	0.384
HDL cholesterol (mg/dL)	36 (10)	34 (10)	0.003
LDL cholesterol (mg/dL)	111 (63)	108 (80)	0.522
Total cholesterol (mg/dL)	184 (49)	176 (39)	0.046
Triglycerides (mg/dL)	215(295)	211 (131)	0.856

SD: standard deviation; NHW: non-Hispanic White; BMI: body mass index; CVD: cardiovascular diseases; TZD: thiazolidinediones; DBP: diastolic blood pressure; SBP: systolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

Data are number of participants and (percent) or means and (SD).

<sup>a</sup>Updated Charlson Comorbidity Index.

significant trend for increasing mortality risk with HbA1c variability, HbA1c variability measures were generally weaker predictors of all-cause mortality than fasting glucose measures of variability.

In Model 2 adjusting for multiple baseline risk factors, the variables' cumulative mean fasting glucose, cumulative maximum fasting glucose, glucose measures (glucose and HbA1c) just prior to death, and log(CV) and ARV of fasting glucose were all significant risk factors (p < 0.05) for all-cause death (Table 2). After additionally adjusting for cumulative mean HbA1c or glucose (Model 3), both fasting glucose and HbA1c variability measures were still significant. Interestingly, variability measures, but not standard measures of glucose control, were significant predictors of all-cause mortality, after adjusting for age, baseline risk factors and cumulative mean HbA1c or glucose.

As adverse lifestyle behaviour may confound the association between glucose variation and mortality, we examined whether adverse behaviours contributed to glucose variability and whether this contribution explained the association of glucose variability with mortality. We found that a cumulative (worse) lifestyle score was a modest but statistically significant predictor of increased glycaemic variability (Supplementary Material). However, after additional adjustment for the effects of cumulative lifestyle factors (Model 4), fasting glucose variability remained a significant predictor of all-cause mortality and was weakened by only 2%–4%.



**Figure I.** Hazard ratio (HR) estimates for quintiles of Log(CV)-glucose and ARV-glucose for mortality adjusted for age. Vertical bars shown are the 95% confidence interval (95% CI) associated with HR estimates. \*\*\* Estimated HR in the indicated variability quintile is significantly higher than the HR of lowest variability quintile (quintile 1). Trend test results are presented as the text annotation in the figures. CV: coefficient of variation; ARV: average real variability.

Additional adjustment for severe hypoglycaemia did not reduce the association of fasting glucose variability with all-cause mortality, but reduced significance of HbA1c variability. When excluding participants who experienced severe hypoglycaemia events (n=268), we found no change in the relationships of log(CV) glucose and ARV glucose with all-cause mortality (Table 3).

#### Discussion

Our findings show that during the VADT glucose lowering intervention phase, visit-to-visit variability measures were significantly associated with all-cause mortality. Adjustment for standard risk factors and standard measures of glucose control (e.g. fasting glucose) did not lessen the association. These data indicate that even these relatively simple measures of visit-to-visit variation may provide additional information regarding future mortality risk. Although the number of events was substantially lower, significant associations were found for both CVD specific (n=57) and all other (n=109) mortality endpoints in exploratory analyses (p < 0.05 for both fasting glucose log(CV) and ARV for using Model 4).

These findings are consistent with the growing body of work demonstrating that oscillation of plasma glucose can enhance oxidative stress generation and alter

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 Table 2. Cox proportional hazards model for all-cause mortality.

	Model I	Model 2	Model 3	Model 4
	HR (CI), <i>p</i> -value	HR (CI), <i>p</i> -value	HR (CI), <i>p</i> -value	HR (CI), <i>p</i> -value
Blood glucose				
Cum-Mean glucose	1.179 (1.033, 1.347), <b>0.015</b>	1.139 (0.992, 1.308), 0.066	I	I
Cum-Max glucose	1.224 (1.082, 1.383), <b>0.002</b>	1.165 (1.026, 1.322), <b>0.019</b>	1.147 (0.945, 1.391), 0.165	Ι
Prior glucose	1.159 (1.013, 1.327), <b>0.032</b>	1.162 (1.012, 1.334), <b>0.033</b>	1.115 (0.934, 1.331), 0.228	Ι
Log(CV) glucose	1.407 (1.197, 1.655), <b>&lt;0.001</b>	1.380 (1.160, 1.641), <b>&lt;0.001</b>	1.362 (1.146, 1.619), <b>&lt;0.001</b>	1.326 (1.115, 1.577), <b>0.002</b>
ARV glucose	1.272 (1.115, 1.451), <0.001	1.232 (1.070, 1.418), <b>0.004</b>	1.218 (1.058, 1.403), <b>0.006</b>	1.186 (1.028, 1.369), <b>0.020</b>
HbAlc				
Cum-Mean HbA1c	1.083 (0.947, 1.238), 0.243	1	I	I
Cum-Max HbA1c	1.128 (0.989, 1.288), 0.074	1	I	Ι
Prior HbAIc	0.951 (0.816, 1.108), 0.519	I	I	Ι
Log(CV) HbA1c	1.244 (1.069, 1.449), <b>0.005</b>	1.213 (1.034, 1.424), <b>0.018</b>	1.215 (1.016, 1.454), <b>0.033</b>	1.194 (0.997, 1.430), 0.054
ARV HbAIc	1.211 (1.059, 1.384), <b>0.005</b>	1.196 (1.038, 1.382), <b>0.014</b>	1.201 (1.021, 1.421), <b>0.028</b>	1.174 (0.996, 1.384), 0.056

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Data are HRs, 95% CI and *p*-values estimated by Cox proportional hazards model for all-cause mortality. Glycaemic control variables that were significant in age-adjusted models (Model 1) were further adjusted for ethnicity (NHW or not), diabetes duration, prior CVD event, baseline diastolic blood pressure, baseline HDL cholesterol, baseline total cholesterol and Charlson comorbidity index (Model 2). The remaining significant glycaemic variation variables in Model 2 were additionally adjusted for the cumulative mean of glucose or HbA1c, respectively, in Model 3. In Model 4, we additionally adjusted for cumulative adverse lifestyle factors. *p*-values in bold font show significant (*p*-value < 0.05) risk for the primary outcome. Note that in Model 2. The remaining significant 3. In Model 4, we additionally adjusted for cumulative adverse lifestyle factors. *p*-values in bold font show significant (*p*-value < 0.05) risk for the primary outcome.

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Variables	Analysis with additional cumulative	Analysis excluding patients				
	severe nypogiycaemia adjustment	naving severe nypogiycaemia				
	Model 4 + severe hypo	Model 4				
	HR (CI), p-value	HR (CI), p-value				
Blood glucose						
Log(CV) glucose	1.316 (1.104, 1.568), <b>0.003</b>	1.451 (1.193, 1.766), <b>&lt;0.001</b>				
ARV glucose	1.177 (1.017, 1.361), <b>0.029</b>	1.272 (1.080, 1.498), <b>0.004</b>				
HbAlc						
Log(CV) HbA1c	1.188 (0.992, 1.423), 0.061	1.276 (1.049, 1.553), <b>0.015</b>				
ARV HbA1c	1.169 (0.991, 1.379), 0.064	1.251 (1.049, 1.492), <b>0.013</b>				

 Table 3. Cox proportional hazards model for all-cause mortality, after accounting for severe hypoglycaemia.

CV: coefficient of variation; ARV: average real variability; HbA1c: glycated haemoglobin; HR: hazard ratio; CI: confidence interval.

Data are HR, 95% CI and *p*-values estimated by Cox proportional hazards model for all-cause mortality. Left panel shows results by additionally adjusting for cumulative hypoglycaemia in Model 4, while right panel shows results when participants with severe hypoglycaemia were excluded. *p*-values in bold font show significant (*p*-value <0.05) risk for the primary outcome. A severe hypoglycaemia episode was defined as 'incomplete loss of consciousness that requires assistance' or 'complete loss of consciousness' occurring since the last visit.  $a_n = 268$ .

endothelial function more than stable elevated levels of glucose.<sup>28,34,35</sup> This suggests that the pattern of glucose control, not just the absolute levels, may also be a determinant of disease risk.

The VADT was a large, carefully conducted trial that provides a more carefully defined and better characterized cohort than was examined in most previously reported studies.<sup>11,19</sup> This allows us both confidence in the 'fasting' nature of blood draws and in the estimates for very important potential confounders such as hypoglycaemia, comorbidity and unhealthy lifestyle behaviour; factors that have infrequently been considered (and never altogether) in analyses. The persistence of glucose variation measures as predictors of all-cause mortality after accounting for these variables provides further support for their unique and clinical importance. In addition, there were many visits over the nearly 7 years of follow-up providing many glucose measures for a robust estimate of long-term visit-to-visit variation. As the VADT was a randomized study of glucose treatment intensity (not different medication classes), participants' diabetes medications were quite similar overall, removing an important potential contributor to glucose variation and outcomes that were less readily addressed in prior observational studies. In contrast to the sub-analysis of ADVANCE,18 this analysis was not limited to the more intensively treated arm, providing a complementary whole cohort analysis that helps make these findings more generalizable.

Our study has several limitations. The typical participant in the VADT was older, predominantly male and at high CVD risk. These results do however support the findings reported from ADVANCE,<sup>18</sup> which included a more diverse set of T2D participants. We were not able to estimate daily glucose variation as that requires more extensive collection of daily glucose measures than was conducted within the VADT. This within day glycaemic variation could add to, or perhaps account for, the effects of visit-tovisit variation. Finally, there are potentially other unmeasured variables, including other adverse lifestyle behaviours, that may account for some of the relationship between glucose variation and mortality.

In conclusion, our study finds a strong association between higher visit-to-visit glycaemic variability and increased risk of mortality during the VADT that is independent of other traditional risk factors. These associations persist even when accounting for the increased risk for severe hypoglycaemia that accompanies greater glucose variation. These results greatly strengthen the growing body of evidence supporting the importance of glycaemic variation in diabetes complications and suggest that efforts to improve glucose control in patients may need to consider how these strategies influence glucose fluctuation.

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# Data availability

The datasets used and/or analysed during this study are available from the corresponding author on reasonable request as permitted by VA guidance.

#### **Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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#### Supplemental material

Supplemental material for this article is available online.

#### **ORCID iD**

Jin J Zhou (i) https://orcid.org/0000-0001-7983-0274

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**Original Article** 

# Association of glucagon-to-insulin ratio and nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus

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Myint Aung Moh Moh<sup>1</sup>, Chan-Hee Jung<sup>2</sup>, Bora Lee<sup>3</sup>, Dughyun Choi<sup>2</sup>, Bo-Yeon Kim<sup>2</sup>, Chul-Hee Kim<sup>2</sup>, Sung-Koo Kang<sup>2</sup> and Ji-Oh Mok<sup>2</sup>

#### Abstract

**Objective:** The aim of this study is to investigate the association between glucagon-to-insulin ratio and the presence of nonalcoholic fatty liver disease on ultrasonography in participants with type 2 diabetes mellitus.

**Research design and methods:** This cross-sectional study was performed with data obtained from 172 participants with type 2 diabetes mellitus admitted to a University hospital of Korea. Participants were assessed for serum fasting and postprandial serum glucagon-to-insulin ratio and divided into tertiles. Nonalcoholic fatty liver disease was defined as ultrasonographically detected fatty liver.

**Results:** Prevalence of nonalcoholic fatty liver disease was significantly decreased across tertile of fasting and postprandial glucagon-to-insulin ratio (p=0.009 for trend, p=0.001 for trend, respectively). Lower glucagon-to-insulin ratio was significantly associated with the presence of nonalcoholic fatty liver disease even after adjustment for potential confounding variables [fasting glucagon-to-insulin ratio: odds ratio (95% confidence interval), 2.68 (1.08–6.86)], postprandial glucagon-to-insulin ratio: [2.72 (1.03–7.35)]. The participants in the lowest tertile of fasting glucagon-to-insulin ratio had higher body mass index, visceral fat thickness, subcutaneous fat thickness, homeostasis model assessment–insulin resistance and shorter duration of diabetes mellitus.

**Conclusion:** This study suggests that lower glucagon relative insulin may be independently associated with nonalcoholic fatty liver disease in participants with type 2 diabetes.

#### **Keywords**

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Glucagon, insulin, glucagon-to-insulin ratio, nonalcoholic fatty liver disease, type 2 diabetes mellitus, insulin resistance

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# Introduction

The pathophysiology of type 2 diabetes mellitus (T2DM) is characterized not only by insulin resistance and  $\beta$ -cell dysfunction but also by relative or absolute hyperglucagonaemia.<sup>1</sup> Recently, the role of glucagon has received much attention as an important additional contributor of glucose control and treatment target of antidiabetic agent.<sup>2,3</sup> Addressing glucagon seems an attractive treatment for T2DM either by suppression of glucagon or by blocking glucagon receptor.<sup>4</sup>

In addition to its effects on glucose metabolism, glucagon is known to exert effects on lipid metabolism.<sup>5</sup> Glucagon action was shown to be essential for multiple pathways regulating lipid homeostasis and leading to reduced lipogenesis.<sup>6</sup> Reduced glucagon action is associated with the development of fatty liver, and exogenous glucagon administration reduces fatty liver in human and animal studies.<sup>7–9</sup> On the contrary, some experimental diabetes settings revealed that attenuation of glucagon action

#### **Corresponding author:**

Ji-Oh Mok, Division of Endocrinology and Metabolism, Department of Internal Medicine, Soonchunhyang University College of Medicine, Soonchunhyang University Bucheon Hospital, 170 Jomaru-ro, Wonmi-gu, Bucheon-si 420-767, Gyeonggi-do, Republic of Korea. Email: hanna@schmc.ac.kr

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Department of Medicine, University of Medicine 2, Yangon, Yangon, Myanmar

<sup>&</sup>lt;sup>2</sup>Division of Endocrinology and Metabolism, Department of Internal Medicine, Soonchunhyang University College of Medicine, Soonchunhyang University Bucheon Hospital, Bucheon, Republic of Korea <sup>3</sup>Department of Biostatistics, Soonchunhyang University College of Medicine, Soonchunhyang University Bucheon Hospital, Bucheon, Republic of Korea

using glucagon receptor knockout mice was associated with reduction in hepatic steatosis.<sup>10</sup>

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and is increasingly diagnosed worldwide.<sup>11</sup> Those with T2DM appear to have an increased risk of developing NAFLD and have a poor hepatic prognosis compared with individuals without T2DM.<sup>12,13</sup> It is well acknowledged that T2DM and NAFLD share a common pathogenic mechanism of insulin resistance.<sup>14,15</sup> On the other hand, some studies have proposed a role of glucagon in NAFLD independent of insulin resistance (IR), but evidence was usually from preclinical or animal studies.<sup>16</sup>

Since glucagon secretion is highly affected by insulin, and the disproportionate changes of the two hormones are clearly revealed, it might make sense to consider glucagon relative to insulin as a glucagon-to-insulin ratio (GI ratio) instead of assessing each absolute value separately.1-3,17 Previous studies have demonstrated that an increase in glucagon concentration and GI ratio reflects hyperglycaemia and degree of glycaemic control in individuals with T2DM.<sup>18-21</sup> Other studies have revealed a role of GI ratio in pancreatic cancer-related diabetes mellitus (DM).22,23 Although glucagon relative to insulin is receiving much attention recently, there are little data regarding the association between GI ratio and NAFLD independent of insulin resistance in individuals with T2DM. There is a possibility that liver lipid infiltration in T2DM could be associated with low glucagon relative to insulin because it may be led by glucagon action reducing lipogenesis. Therefore, the aim of this study is to investigate the hypothesis that lower GI ratio is associated with the presence of NAFLD.

### **Methods**

#### Study design and subjects

Among 230 participants with T2DM who were admitted for glucose control to the Endocrinology Division of Soonchunhyang University Bucheon Hospital from April 2015 to June 2017, participants were eligible if they had no history or clinical evidence of chronic liver disease or cirrhosis, no positive test for hepatitis B or hepatitis C, no medication associated with hepatotoxicity and no history of alcohol consumption. 'No history of alcohol consumption' was defined as less than 30 g alcohol/day for men and less than 20 g alcohol/day for women.24,25 In addition, participants with type 1 diabetes, those older than 80 years of age, those with inflammatory bowel disease or gut resection except appendectomy, those lacking fasting glucagon and insulin data and those lacking liver ultrasonography (US) data were excluded. Finally, 172 participants were included for analysis in this cross-sectional study. In total, 12 of the 172 participants were lacking postprandial glucagon data. We reviewed detailed demographic, biochemical and clinical data and treatment history using medical records. The smoking status of the subjects was classified as non-smoker or current smoker. All participants were informed of the purpose of the study, and their consent was obtained. The study was approved by the Institute Review Board of Soonchunhyang University School of Medicine, Bucheon Hospital.

### Anthropometric and biochemical measurements

Height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. Body mass index (BMI) was calculated as body weight (kg) divided by height squared (square metres). Blood samples were collected after overnight fasting. HbA1c was measured by ion exchange highperformance liquid chromatography (Bio-Rad, Hercules, CA, USA). The methodology was aligned with the Diabetes Control and Complications Trial and National Glycohemoglobin Standardization Program standards.<sup>26</sup> Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) were determined using the liquid enzymatic method with an automatic biochemical analyzer (7600-110; Hitachi Inc., Tokyo, Japan) and high-density lipoprotein cholesterol (HDL-C) was measured by the selective inhibition method. Estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) study equation.

Participants underwent a meal test with measurements of plasma glucose, insulin and glucagon concentrations at 0 and 30 min. Serum insulin was measured using an immunoradiometric assay kit (DIAsource, Belgium). Fasting and postprandial samples for plasma glucagon were collected and analyzed using a radioimmunoassay kit (MP Biomedical, CA, USA).

The insulin resistance (IR) status was evaluated by the homeostasis model assessment–insulin resistance (HOMA-IR) index. The HOMA-IR was calculated by the following formula: [fasting insulin ( $\mu$ IU/mL)×fasting plasma glucose (mmol/L)]/22.5.

# Evaluation of fatty liver by US

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Liver US was carried out by experienced radiologists. The diagnosis of hepatic steatosis was made on the basis of characteristic sonographic features: that is, diffuse hyperechogenicity of the liver; increased liver contrast compared to kidney; vascular blurring, mainly of portal veins and attenuation of echogenic level in a deep seated area.<sup>25</sup>

Sonographic measurement of abdominal fat thickness was performed using high-resolution B-mode US by a single experienced investigator.<sup>27,28</sup> Subcutaneous fat thickness (SFT) and visceral fat thickness (VFT) were measured in the region 1 cm above the umbilicus using a 12-MHz linear-array probe and a 3.5-MHz convex-array probe,

respectively. SFT was defined as the maximal thickness of the fat tissue layer between the skin-fat interface and the linea alba. VFT was defined as the distance between the anterior wall of the aorta and the posterior aspect of the rectus abdominis muscle perpendicular to the aorta. The intra-observer technical error of measurement was 1.4%–2.3% for VFT and 1.1%–1.7% for SFT.

## Statistical analysis

Data are reported as mean  $\pm$  standard deviation (SD) for continuous variables or as number of participants (percentage) for categorical variables. Differences in demographic and clinical characteristics according to the tertile of GI ratio were evaluated by one-way analysis of variance (ANOVA) or Kruskal–Wallis test for continuous variables and chi-square test for categorical variables. To evaluate the linear trend of the prevalence of NAFLD according to GI ratio tertile, *p* values were calculated by Jonckheere– Terpstra linear trend test for continuous variables and Mantel–Haenszel's linear-by-linear association test for categorical variables.

The correlations of serum GI ratio and other clinical variables were assessed by Spearman's rank correlation coefficient. The odds ratio (OR) was used as a measure of the association between serum GI ratio and the presence of NAFLD in multivariate logistic regression analysis. To compute ORs of serum GI ratio, several models were set up and adjusted for potential confounders determined through the result of the group comparison and the assessment of multicollinearity by generalized variance inflation factor (GVIF).

A two-tailed *p* value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS (version 14.0; SPSS, Inc., Chicago, IL) and R (version 3.1.3, The R Foundation for Statistical Computing, Vienna, Austria).

# Results

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# Clinical characteristics of participants according to GI ratio

The general characteristics of the study population are presented in Table 1. There was no significant difference by sex. The mean age of the study subjects was 57 years, and the mean duration of DM was 7.5 years. Among all of the participants, 87 (51%) had been diagnosed with NAFLD.

The participants were divided into three groups according to GI ratio. The clinical parameters according to fasting GI ratio tertile and postprandial GI ratio tertile are shown in Tables 2 and 3, respectively. The mean levels of BMI, VFT, SFT, HOMA-IR and eGFR were significantly decreased with increasing tertiles, and duration of DM and fasting and postprandial glucagon was significantly **Table I.** The clinical characteristics and laboratory findings of the study populations.

Variable	Total			
	(N=172)			
Age (years)	57±16			
Men	86 (50%)			
Duration of DM (years)	$7.5\pm8.6$			
Current smoking (%)	31 (18.0%)			
Alcohol (%)	38 (22%)			
BMI (kg/m <sup>2</sup> )	$\textbf{24.9} \pm \textbf{4.4}$			
VFT (cm)	$\textbf{43.4} \pm \textbf{20.6}$			
SFT (cm)	$13.8\pm5.6$			
Insulin (fasting, μIU/mL)	$8.8\pm 6.1$			
Insulin (30 min; μIU/mL)	$16.8\pm13.1$			
Glucagon (fasting; pg/mL)	$244\pm198$			
Glucagon (30 min; pg/mL)	$297\pm191$			
HOMA-IR	$\textbf{4.0} \pm \textbf{3.0}$			
HbAIc (mmol/mol; %)	91 $\pm$ 9 (10.6 $\pm$ 2.5)			
AST (IU/L)	$27\pm24$			
ALT (IU/L)	$29\pm29$			
eGFR (mL/min/1.73 m <sup>2</sup> ) (MDRD)	$\textbf{66.3} \pm \textbf{25.8}$			
DPP4 inhibitor	42 (31.8%)			
Total cholesterol (mg/dL)	$168 \pm 51$			
LDL-cholesterol (mg/dL)	$97\pm39$			
HDL-cholesterol (mg/dL)	$43 \pm 14$			
Triglycerides (mg/dL)	$178\pm202$			
NAFLD (%)	877 (51%)			
Treatment modality				
No medication	63 (36.7%)			
OHA only	57 (33.1%)			
Insulin only	32 (18.6%)			
OHA + insulin	20 (11.6%)			

MDRD: Modification of Diet in Renal Disease; DM: diabetes mellitus; BMI: body mass index; VFT: visceral fat thickness; SFT: subcutaneous fat thickness; HOMA-IR: homeostasis model assessment-insulin resistance; HbA1c: glycated haemoglobin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; eGFR: estimated glomerular filtration rate; DPP4: dipeptidyl peptidase 4; LDL: low-density lipoprotein; HDL: highdensity lipoprotein; NAFLD: nonalcoholic fatty liver disease; OHA: oral hypoglycaemic agent.

Data were reported as mean  $\pm$  standard deviation for continuous variables and n(%) for categorical variables.

increased across fasting GI ratio tertiles (p=0.002, p=0.006, p<0.001, p<0.001, p=0.039, p=0.036, p<0.001 and p<0.001, respectively; Table 2). The prevalence of fatty liver was significantly decreased across GI ratio tertiles (p=0.009; Table 2). Dipeptidyl peptidase 4 (DPP4) inhibitor treatment was comparable among the three groups (p=0.32; Table 2). The mean levels of fasting and postprandial insulin, HbA1c, AST, ALT and lipid profiles were comparable among the groups (Table 2). Similar results were also shown according to postprandial GI ratio tertile except for some clinical variables (Table 3). The mean levels of HbA1c, HDL-C and postprandial glucagon

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Table 2.	The clinical	characteristics and	laboratory	y findings accord	ing to the t	ertile of fastin	g glucagon-to-insulin ratio.
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Variable	Fasting glucagon-to-insul		p for		
	Tertile   (<18.87)	Tertile 2 (18.87–37.73)	Tertile 3 (>37.73)	trend	
N	56	57	58		
Age (years)	54.0 [50.5; 65.0]	60.0 [47.0; 73.0]	56.5 [39.8; 72.0]	0.858	
Men	23 (41.1%)	33 (57.9%)	30 (51.7%)	0.261	
Duration of DM (year)	1.0 [0.0; 10.0]	5.0 [0.0; 13.0]	6.0 [1.0; 15.0]	0.036	
Current smoking (%)	7 (12.5%)	10 (17.5%)	5 (8.9%)	0.528	
Alcohol (%)	13 (23.2%)	18 (31.6%)	7 (12.5%)	0.147	
BMI (kg/m <sup>2</sup> )	25.9 ± 4.6	25.0 ± 3.7	23.6 ± 4.5	0.002	
VFT (cm)	46.2 [28.6; 54.8]	45.2 [34.4; 56.3]	32.8 [23.6; 44.1]	0.006	
SFT (cm)	14.8 [11.4; 20.2]	13.6 [10.6; 17.4]	11.0 [7.8; 14.5]	<0.001	
Insulin (fasting; µIU/mL)	11.4 [8.5; 15.2]	7.8 [6.1; 10.0]	4.1 [1.7; 6.4]	0.214	
Insulin (30 min; µIU/mL)	20.7 [12.9; 29.0]	14.3 [9.2; 21.5]	7.5 [3.6; 12.4]	0.214	
Glucagon (fasting; pg/mL)	134.5 [109.7; 174.5]	216.0 [157.0; 286.0]	277.5 [178.0; 422.3]	<0.001	
Glucagon (30 min; pg/mL)	208.7 [143.0; 246.8]	273.0 [222.0; 350.0]	317.8 [222.0; 455.0]	< 0.00 l	
HOMA-IR	4.7 [3.2; 7.0]	3.1 [2.1; 6.0]	2.1 [0.8; 3.3]	<0.001	
HbAIc (mmol/mol; %)	88±9 (10.2±2.2)	93 ± 9 (10.7 ± 2.6)	97 ± 9 (11.0 ± 2.6)	0.099	
AST (IU/L)	21 [17; 34.5]	18 [15; 24]	20 [14.3; 31.5]	0.441	
ALT (IU/L)	22 [15.5; 44]	17 [13; 25.3]	18 [13; 28.8]	0.565	
eGFR (mL/min/1.73 m <sup>2</sup> ) (MDRD)	75.0 [59.3; 86.0]	58.9 [48.4; 75.3]	68.9 [40.9; 81.6]	0.039	
Metformin	18 (45%)	19 (39.6%)	19 (38%)	0.786	
DPP4 inhibitor	15 (41.7%)	13 (31.0%)	11 (24.4%)	0.32	
SGLT2 inhibitor	I (I.8%)	2 (3.4%)	0`´´	0.369	
Insulin	10 (17.5%)	19 (32.8%)	23 (40.4%)	0.026	
Total cholesterol (mg/dL)	169.4±54.0	168.3 ± 54.3	165.1 ± 45.9	0.635	
LDL-cholesterol (mg/dL)	95.0 [72.0; 123.0]	97.0 [69.0; 118.5]	87.0 [58.0; 115.0]	0.188	
HDL-cholesterol (mg/dL)	40.0 [34.5; 47.0]	39.0 [35.5; 49.5]	43.0 [35.5; 50.5]	0.302	
Triglycerides (mg/dL)	122.0 [85.0; 190.5]	139.5 [97.5; 228.0]	124.0 [72.0; 180.0]	0.491	
NAFLD (%)	34 (70.8%)	32 (66.7%)	21 (42.0%)	0.009	
Treatment modality				0.095	
No medication	24 (41.2%)	21 (36.2%)	18 (31.6%)		
OHA only	21 (36.8%)	18 (31.0%)	18 (31.6%)		
Insulin only	8 (14%)	13 (22.4%)	11 (19.3%)		
OHA + insulin	4 (7%)	6 (10.3%)	10 (17.5%)		

MDRD: Modification of Diet in Renal Disease; DM: diabetes mellitus; BMI: body mass index; VFT: visceral fat thickness; SFT: subcutaneous fat thickness; HOMA-IR: homeostasis model assessment-insulin resistance; HbA1c: glycated haemoglobin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; eGFR: estimated glomerular filtration rate; DPP4: dipeptidyl peptidase 4; SGLT2: sodium-glucose cotransporter 2; LDL: low-density lipoprotein; HDL: high-density lipoprotein; NAFLD: nonalcoholic fatty liver disease; OHA: oral hypoglycaemic agent.

Data were reported as mean  $\pm$  standard deviation or median [interquartile range (IQR)] for continuous variables and n(%) for categorical variables. *p* values were calculated by Jonckheere–Terpstra linear trend test for continuous variables and Mantel–Haenszel's linear-by-linear association test for categorical variables.

steadily increased and postprandial insulin level gradually decreased across postprandial GI ratio tertiles (p=0.003, p=0.029, p<0.001 and <0.001, respectively). The mean eGFR levels were not different in postprandial GI ratio tertile groups (p=0.334; Table 3).

# Bivariate correlations of serum GI ratio with clinical variables

The serum fasting and postprandial GI ratios were negatively correlated with BMI (r=-0.29, p < 0.001, r=-0.307, p < 0.001), VFT (r=-0.27, p=0.001, r=-0.331, p < 0.001),

SFT (r=-0.325, p<0.001, r=-0.231, p=0.007), fasting and postprandial insulin (r=-0.716, p<0.001, r=-0.531, p<0.001, r=-0.46, p<0.001, r=-0.818, p<0.001), HOMA-IR (r=-0.56, p<0.001, r=-0.312, p<0.001) and ALT (r=-0.164, p=0.032, r=-0.251, p=0.001) and positively correlated with fasting and postprandial glucagon (r=0.538, p<0.001, r=393, p<0.001, r=0.413, p<0.001, r=0.507, p<0.001; Table 4). Duration of DM was positively correlated with fasting GI ratio but not postprandial GI ratio (r=158, p=0.039, r=0.091, p=0.253). HbA1c and HDL-C were positively correlated with postprandial GI ratio but not fasting GI ratio (r=0.167, p=0.03,

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Variable	Postprandial glucagon-to-insulin ratio tertile						
	Tertile   (<13.40)	Tertile 2 (13.40–29.69)	Tertile 3 (>29.69)	trend			
N	53	53	54				
Age (years)	56.0 [46.0; 72.0]	58.0 [46.0; 67.0]	56.5 [42.0; 72.0]	0.786			
Men	26 (49.1%)	30 (56.6%)	26 (48.1%)	0.921			
Duration of DM (years)	3.0 [0.0; 10.0]	4.0 [0.0; 13.0]	5.0 [0.0; 15.0]	0.653			
Current smoking (%)	6 (11.3%)	10 (19.2%)	6 (11.3%)	0.969			
Alcohol (%)	14 (26.4%)	16 (30.8%)	6 (11.3%)	0.057			
BMI (kg/m <sup>2</sup> )	$\textbf{26.2} \pm \textbf{4.9}$	$24.7\pm3.4$	$\textbf{23.5} \pm \textbf{4.2}$	0.002			
VFT (cm)	47.1 [37.0; 53.9]	44.3 [30.9; 54.2]	32.8 [23.6; 42.3]	0.001			
SFT (cm)	13.3 [10.7; 20.2]	3.9 [  . ;  6.6]	.8 [8.0;  4.4]	0.018			
Insulin (fasting; μIU/mL)	9.1 [7.3; 13.7]	7.6 [6.0; 11.1]	4.5 [3.2; 7.8]	0.217			
Insulin (30 min; µIU/mL)	23.8 [18.5; 29.0]	3.2 [ 0.4;  6.3]	6.0 [2.2; 9.6]	<0.001			
Glucagon (fasting; pg/mL)	150.0 [119.5; 216.2]	199.0 [132.0; 282.3]	254.8 [167.8;415.0]	0.223			
Glucagon (30 min; pg/mL)	207.5 [135.0; 242.0]	273.0 [205.3; 341.0]	335.5 [222.0; 476.0]	<0.001			
HOMA-IR	4.2 [2.4; 7.1]	3.2 [2.2; 5.4]	2.5 [1.0; 3.9]	0.002			
HbAIc (mmol/mol; %)	84 $\pm$ 9 (9.8 $\pm$ 2.0)	95 $\pm$ 9 (10.8 $\pm$ 2.4)	99 $\pm$ 9 (11.2 $\pm$ 2.7)	0.003			
eGFR (mL/min/1.73 m <sup>2</sup> ) (MDRD)	66.3 [54.0; 85.4]	65.2 [48.4; 78.5]	67.5 [47.0; 82.8]	0.334			
AST (IU/L)	21 [17–32.8]	19 [15–29]	19 [14–25]	0.986			
ALT (IU/L)	21.5 [15.3–42]	17.5 [13–35]	17 [12–23.5]	0.245			
Metformin	22 (50%)	15 (38.5%)	15 (33.3%)	0.263			
DPP4 inhibitor	17 (44.7%)	9 (26.5%)	10 (23.8%)	0.094			
SGLT2 inhibitor	0	3 (5.6%)	0	0.05			
Insulin	( 8.9%)	17 (31.5%)	20 (37.7%)	0.094			
Total cholesterol (mg/dL)	$168.0\pm57.3$	$166.1 \pm 52.8$	$165.2\pm43.0$	0.955			
LDL-cholesterol (mg/dL)	92.5 [69.0; 123.0]	95.5 [68.5; 119.5]	89.0 [61.0; 113.0]	0.334			
HDL-cholesterol (mg/dL)	38.0 [35.0; 46.0]	38.0 [32.0; 48.0]	46.5 [37.0; 52.0]	0.029			
Triglycerides (mg/dL)	126.5 [80.0; 201.5]	153.0 [105.0; 213.0]	112.5 [82.0; 167.0]	0.277			
NAFLD (%)	32 (69.6%)	33 (68.8%)	16 (39.0%)	0.001			
Treatment modality				0.991			
No medication	18 (34.0%)	20 (37.1%)	21 (39.6%)				
OHA only	20 (37.7%)	19 (35.2%)	14 (26.4%)				
Insulin only	9 (17.0%)	12 (22.2%)	10 (18.9%)				
OHA + insulin	6 (11.3%)	3 (5.6%)	8 (15.1%)				

Table 3. The clinical characteristics and laboratory findings according to the tertile of postprandial glucagon-to-insulin ratio.

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MDRD: Modification of Diet in Renal Disease; DM: diabetes mellitus; BMI: body mass index; VFT: visceral fat thickness; SFT: subcutaneous fat thickness; HOMA-IR: homeostasis model assessment-insulin resistance; HbA1c: glycated haemoglobin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; eGFR: estimated glomerular filtration rate; DPP4: dipeptidyl peptidase 4; SGLT2: sodium-glucose cotransporter 2; LDL: low-density lipoprotein; HDL: high-density lipoprotein; NAFLD: nonalcoholic fatty liver disease; OHA: oral hypoglycaemic agent.

Data were reported as mean  $\pm$  standard deviation or median [interquartile range (IQR)] for continuous variables and n(%) for categorical variables.

p values were calculated by Jonckheere–Terpstra linear trend test for continuous variables and Mantel–Haenszel's linear-by-linear association test for categorical variables.

r=0.199, p=0.012, r=0.141, p=0.066, r=0.124, p=0.11) and AST was negatively correlated with only postprandial GI ratio (r=-0.208, p=0.008). No significant correlation was seen between eGFR, TC, LDL-C and TG and fasting or postprandial GI ratio (Table 4).

# Comparison of prevalence of NAFLD across GI ratio tertiles

Prevalence of NAFLD according to fasting or postprandial GI ratio tertile showed statistically significant differences

(*p* for trend=0.009, p=0.001, respectively), with a significant decrease in the prevalence of NAFLD with GI ratio tertile (70.8% vs 66.7% vs 42% in fasting GI ratio tertile; 69.6% vs 68.8% vs 39% in postprandial GI ratio tertile; Figure 1).

# Multiple logistic regression analysis of the association of GI ratio with presence of NAFLD

Multivariate logistic regression analysis was used to calculate ORs for NAFLD as a function of GI ratio tertile

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Variable	Fasting glucagon-to-insulin ratio	Postprandial glucagon-to-insulin ratio			
	r (p value)	r (p value)			
Age (years)	0.001 (0.988)	-0.028 (0.725)			
Duration of DM (years)	0.158 (0.039)	0.091 (0.253)			
BMI (kg/m <sup>2</sup> )	-0.29 (<0.001)	-0.307 (<0.001)			
VFT (cm)	-0.27 (0.001)	-0.331 (<0.001)			
SFT (cm)	-0.325 (<0.001)	-0.231 (0.007)			
HOMA-ÍR	-0.56 (<0.001)	-0.312 (<0.001)			
HbAIc (mmol/mol)	0.141 (0.066)	0.167 (0.03)			
eGFR (mL/min/1.73 m <sup>2</sup> ) (MDRD)	-0.121 (0.115)	-0.062 (0.433)			
AST (IU/L)	-0.134 (0.08)	-0.208 (0.008)			
ALT (IU/L)	-0.164 (0.032)	-0.251 (0.001)			
Total cholesterol (mg/dL)	-0.012 (0.876)	-0.018 (0.826)			
LDL-cholesterol (mg/dL)	-0.081 (0.3)	-0.08 (0.322)			
HDL-cholesterol (mg/dL)	0.124 (0.11)	0.199 (0.012)			
Triglycerides (mg/dL)	-0.085 (0.275)	-0.138 (0.086)			

Table 4. Correlation of fasting glucagon-to-insulin ratio, postprandial glucagon-to-insulin ratio with other clinical variables.

r: Spearman's correlation coefficient; MDRD: Modification of Diet in Renal Disease; DM: diabetes mellitus; BMI: body mass index; VFT: visceral fat thickness; SFT: subcutaneous fat thickness; HOMA-IR: homeostasis model assessment-insulin resistance; HbA1c: glycated haemoglobin; eGFR: estimated glomerular filtration rate; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDL: low-density lipoprotein; HDL: high-density lipoprotein.





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(Table 5). Individuals with the lowest fasting GI ratio had an OR of 3.35 [95% confidence interval (CI)=1.47–7.93] for NAFLD compared to those with the highest GI ratio after adjusting for age and sex. This significant association remained after adjusting for BMI, DM duration and insulin treatment [OR=2.68 (1.08–6.86)).]. In addition, the OR for presence of NAFLD according to postprandial GI ratio tertile was also evaluated and revealed similar results with those of fasting GI ratio. Individuals with the lowest postprandial GI ratio tertile had an OR of 2.72 [1.03–7.35] for NAFLD compared to those with the highest GI ratio even after adjusting for the above mentioned covariates.

# Discussion

In this study, there was a significant relation between both fasting and postprandial GI ratio and presence of fatty liver. We found that the prevalence of NAFLD was significantly decreased across tertiles of fasting and postprandial GI ratio. This suggests that lower glucagon relative to

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Variable	Model I	Model 2	Model 3	Model 4
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Fasting glucagon-to-ins	ulin-ratio			
Tertile I $(n=56)$	3.35*** (1.47 to 7.93)	3.33 <sup>∞</sup> (1.46 to 7.92)	2.86* (1.22 to 6.9)	2.68* (1.08 to 6.86)
Tertile 2 $(n = 57)$	2.76* (1.23 to 6.4)	2.79* (1.23 to 6.53)	2.59* (1.13 to 6.15)	2.21 (0.91 to 5.45)
Tertile 3 $(n = 58)$	Ref (1.00)	Ref (1.00)	Ref (1.00)	Ref (1.00)
Postprandial glucagon-	to-insulin ratio			· · ·
Tertile I $(n=53)$	3.57** (1.49 to 8.89)	3.56** (1.49 to 8.88)	3.48** (1.42 to 8.86)	2.72* (1.03 to 7.35)
Tertile 2 $(n=53)$	3.44** (1.45 to 8.44)	3.44** (1.45 to 8.46)	3.38** (1.39 to 8.52)	2.53 (0.99 to 6.64)
Tertile 3 $(n=54)$	Ref (1.00)	Ref (1.00)	Ref (1.00)	Ref (1.00)

**Table 5.** Relative risk for fatty liver according to glucagon-to-insulin ratio tertile.

OR: odds ratio; CI: confidence interval; BMI: body mass index.

Model I: no adjustment.

Model 2: Model I plus age, sex.

Model 3: Model 2 plus diabetes mellitus duration and insulin treatment.

Model 4: Model 3 plus overweight (BMI≥23).

\*p<0.05; \*\*p<0.01.

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insulin may be independently associated with NAFLD in individuals with T2DM.

The pathophysiologic role of glucagon in development of T2DM has been recognized during the last couple of decades; in recent years, it has been attracting much interest as an important treatment target of antidiabetic agents. The reduction of glucagon level is one of the main mechanisms of action of some antidiabetic drugs for T2DM.<sup>4</sup>

By modulating the relative concentrations of glucagon and insulin, the alpha and beta cells of the pancreas control glucose metabolism. Since glucagon secretion is highly affected by insulin, and absolute insulin and glucagon levels have not been determined in subjects with diabetes, it may make sense to consider the GI ratio instead of assessing absolute values.<sup>17</sup> GI ratio describes the significance of GI bipolar axis. Increased GI ratio may reflect insulinopenia or relative hyperglucagonaemic conditions, and decreased GI ratio may reflect hyperinsulinaemia or insulin resistance.

In addition to its effects on glucose metabolism, glucagon is also known to exert effects on lipid metabolism.<sup>7</sup> Glucagon exerts hypolipidaemic effects on hepatocytes and promotes mobilization of hepatic fat in various species and preclinical studies.<sup>6,7,29</sup> Exogenous glucagon administration reduced fatty liver in human and animal studies.<sup>9,10</sup> In addition, attenuation of glucagon receptor signalling is predicted to be associated with increased risk of fatty liver.<sup>6,30</sup> Such results have proposed a beneficial role of glucagon in NAFLD, whereas conflicting results have also been reported.<sup>8</sup> An experimental diabetes setting revealed that attenuation of glucagon action using glucagon receptor knockout mice has been associated with reduction in hepatic steatosis with or without compensatory increased plasma glucagon.<sup>11</sup>

Although NAFLD is considered a hepatic manifestation of metabolic syndrome and its main pathogenesis is based on insulin resistance, which is an essential component of T2DM, a significant role of glucagon in NAFLD has also been suggested.<sup>15,16</sup> However, research regarding the relationship of glucagon and NAFLD in subjects with T2DM is scarce and inconsistent. Since glucagon reduces lipogenesis by multiple mechanisms, it was thought that reduction of glucagon signalling, that is, via the use of glucagon receptor antagonists, may lead to the unfavourable accumulation of lipids in the liver.<sup>30</sup> Chronic treatment with a glucagon receptor antagonist demonstrated increases in hepatic fat in individuals with T2DM.<sup>31</sup> Anoop et al.<sup>32</sup> reported that the mean values of fasting and postprandial glucagon levels were higher in group of T2DM with NAFLD compared to group of T2DM without NAFLD in 81 Indian men with T2DM. Suppli et al.<sup>33</sup> reported that both normoglycaemic individuals and individuals with T2DM with NAFLD exhibit fasting hyperglucagonaemia compared to similarly grouped individuals without NAFLD. However, whether hyperglucagonaemia is a compensatory consequence of steatosis or directly involved in the pathogenesis of NAFLD remains unanswered.

Nonlinearities in the liver response to the insulin and glucagon stimuli may exist in the physiologic range, and the pattern of their interaction may be very complex.<sup>17</sup> Although more severe insulin resistance was associated with higher fasting glucagon level, less early glucagon suppression and greater late glucagon suppression, the relationship between insulin sensitivity and fasting glucagon concentration was shown to be nonlinear. Therefore, it may make sense to consider glucagon relative to insulin instead of each absolute value. To our knowledge, there is no reported study regarding the relationship of GI ratio and NAFLD in individuals with T2DM. Therefore, we investigated their associations in participants with T2DM and found that the presence of NAFLD was significantly increased in participants with lower GI ratio. Decreased GI

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ratio may reflect hyperinsulinaemia or insulin resistance, but relatively lower glucagon levels were associated with NAFLD. The lower is the ratio, the greater is the likelihood of having NAFLD.

Very recently, a retrospective study using a large Canadian diabetes register investigated changes in ALT levels as a marker of NAFLD among subjects initiated on SGLT2 inhibitors and incretin agents in comparison to a reference control group.34 That study showed that SGLT2 inhibitors were associated with significantly greater reduction in ALT compared to incretin therapies independent of weight and HbA1c change. Ferrannini et al.35 and Hansen et al.36 explained that the opposing effects of SGLT2 inhibitors and incretin agents on insulin-glucagon ratio may be a possible mechanism. Whereas SGLT2 inhibitors lead to glucagon stimulation leading to a fall in insulin-glucagon ratio, incretin agents lead to glucagon suppression and insulin stimulation, resulting in a rise in the insulin-glucagon ratio. Although there were no significant differences in the prescription of SGLT2 inhibitors and DPP4 inhibitors in our study, their study result is in line with our study results and hypothesis. Future works are needed to investigate the mechanistic link regarding GI ratio and NAFLD by randomized, prospective studies comparing drugs such as SGLT2 inhibitors and incretin agents affecting GI ratio in T2DM subjects with NAFLD.

It has been suggested that an increase in GI ratio is an important determinant of the hyperglycaemia seen in individuals with T2DM.<sup>19</sup> Increased GI ratio may reflect insulinopenia or relative hyperglucagonaemia. Consistent with a previous study, this study showed that higher postprandial GI ratio was positively correlated with HbA1c, FBG and HDL-C levels.<sup>20,21</sup> In patients with pancreatic cancer, GI ratio after a 75-g oral glucose challenge was independently correlated with HbA1c level.<sup>22</sup> In addition, appropriate choice of drugs for T2DM can be determined according to the glucagon response or change in absolute glucagon or GI ratio after glucose lowering drugs.<sup>4</sup>

Our study showed that fasting and postprandial GI ratios were negatively correlated with BMI, VFT and SFT. Higher GI ratio representing relative high glucagon level or lower insulin level may suggest less insulin resistance, so it had lower BMI and VFT, and SFT. This is in line with previous findings showing lower GI in participants with higher BMI.20 Physiologically, exogenous glucagon reduces lipoprotein via the glucagon receptor with stimulated fatty acid oxidation and less adiposity.37 However, GI ratio and its relation to adiposity measured by VFT and SFT never have been reported. Recently, only one study revealed an opposing result that high plasma glucagon level correlates with waist-to-hip ratio, suprailiac skinfold thickness and deep subcutaneous abdominal and intraperitoneal adipose tissue depots in nonobese Asian Indian men with T2DM.32 They assessed subcutaneous and visceral

adiposity using whole-body dual-energy X-ray absorptiometry (DEXA) and magnetic resonance imaging (MRI) and its relation with glucagon level not with GI ratio.

This study showed significant difference of mean eGFR according to GI ratio. The role of glucagon in the kidney is known to regulate the GFR, urea excretion and electrolyte excretion.<sup>38</sup> These changes were shown at relatively high doses of glucagon and were more evident in diabetes, possibly due to the modified GI ratio.<sup>39</sup>

The strength of this study is that it is the first report on the association of serum GI ratio and NAFLD as identified on US in participants with T2DM. Nevertheless, there are limitations to our study. First, we cannot determine any causative relationship between GI ratio and NAFLD due to the cross-sectional nature of the study. Second, because most study subjects were hospitalized participants admitted due to poor glucose control, there may be some concern over the accuracy of measurement of glucagon and insulin, and the results may not represent the entire population with diabetes. In addition, there was no comparator group without T2DM in this study. Third, postprandial insulin and glucagon were not measured after oral glucose tolerance test but after a normal meal, which means calorie and nutrient intakes may have varied from subject to subject. Fourth, study subjects with various medications, in particular DPP4 inhibitor, could be another limitation as well, because incretin-based therapies might affect serum level of glucagon.<sup>4</sup> However, statistical significance was still prominent after adjusting for medication including insulin treatment. Fifth, we did not evaluate the incretin hormones that affect glucagon.<sup>40</sup> In addition, the limited number of subjects in the studies increases the risk of type 2 errors. However, previous studies were mainly animal studies or human studies with less than 100 patients. Finally, we assessed NAFLD by ultrasound, which can only detect steatosis involving more than 20%–30% of hepatocytes. In theory, we may have overlooked low-degree steatosis because we did not perform liver biopsies.

In conclusion, this study showed that the fasting and postprandial GI ratios were significantly associated with the presence of NAFLD on US. Our results suggest that lower glucagon relative insulin may be independently associated with NAFLD in participants with type 2 diabetes. We cautiously speculate that GI ratio could be a useful marker for diagnosis of NAFLD in participants with T2DM.

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**Original Article** 

Effects of magnitude of visceral adipose tissue reduction: Impact on insulin resistance, hyperleptinemia and cardiometabolic risk in adolescents with obesity after long-term weight-loss therapy Diabetes & Vascular Disease Research 2019, Vol. 16(2) 196–206 © The Author(s) 2019 Article reuse guidelines: sagepub.com/journals-permissions DOI: 10.1177/1479164118825343 journals.sagepub.com/home/dvr

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Raquel Munhoz da Silveira Campos<sup>1,5</sup>, Deborah Cristina Landi Masquio<sup>2</sup>, Flávia Campos Corgosinho<sup>3</sup>, Danielle Arisa Caranti<sup>4,5</sup>, Aline de Piano Ganen<sup>2</sup>, Lian Tock<sup>6</sup>, Lila Missae Oyama<sup>7,8</sup> and Ana Raimunda Dâmaso<sup>7</sup>

#### Abstract

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**Aim:** To investigate the association between visceral adipose tissue loss and insulin resistance and hyperleptinemia in adolescents with obesity submitted to interdisciplinary weight-loss therapy.

**Methods:** A total of 172 post-pubertal adolescents (body mass index greater than the 95th percentile of the Centers for Disease Control and Prevention reference growth charts) were recruited for the study. The adolescents were assigned to long-term weight-loss therapy. Body composition, visceral and subcutaneous fat, glucose metabolism, lipid profile, hepatic enzymes and leptin concentration were measured. After the therapy, the adolescents were allocated to three different groups according to the tertile of visceral fat reduction.

**Results:** Positive effects on body composition were observed in all analysed groups independent of visceral fat reduction. It was found that visceral fat was an independent predictor of insulin resistance in the investigated population. Obese adolescents who lost a higher proportion of visceral adipose tissue (>1.8 cm) demonstrated improved metabolic and inflammatory parameters twice as much than those who presented smaller losses. Positive correlations between visceral fat reduction and glucose metabolism, lipid profile, hepatic enzymes and homeostasis model assessment of insulin resistance index were demonstrated.

**Conclusion:** The magnitude of the reduction in visceral fat was an independent predictor of insulin resistance, hyperleptinemia and metabolic disorders related to obese adolescents.

#### Keywords

Visceral fat, insulin resistance, obesity, cardiometabolic risk, hyperleptinemia state

<sup>1</sup>Department of Physiotherapy, Therapeutic Resources Laboratory, Universidade Federal de São Carlos (UFSCar), São Carlos, Brazil

<sup>2</sup>São Camilo University Center, São Paulo, Brazil

<sup>3</sup>Universidade Federal de Goiás (UFG), Goiânia, Brazil <sup>4</sup>Post Graduate Program of Interdisciplinary Health Sciences,

Universidade Federal de São Paulo (UNIFESP), Santos, Brazil <sup>5</sup>Department of Biosciences, Universidade Federal de São Paulo

(UNIFESP), Santos, Brazil <sup>6</sup>Weight Science, São Paulo, Brazil

"Weight Science, Sao Faulo, Brazi

 <sup>7</sup>Post Graduate Program of Nutrition, Paulista School of Medicine, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil
 <sup>8</sup>Department of Physiology, Paulista School of Medicine, Universidade Federal de São Paulo, São Paulo, Brazil

#### **Corresponding authors:**

Raquel Munhoz da Silveira Campos, Department of Biosciences, Universidade Federal de São Paulo (UNIFESP), Rua Silva Jardim, 136 - Térreo. Vila Matias Santos, São Paulo 11015020, Brazil.

Email: raquelmunhoz@hotmail.com

Ana Raimunda Dâmaso, Post Graduate Program of Nutrition, Paulista School of Medicine, Universidade Federal de São Paulo (UNIFESP), Rua Marselhesa, 650–Vila Clementino, São Paulo 04020-050, Brazil. Email: ana.damaso@unifesp.br

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### Introduction

In his 1988 Banting Lecture, Dr Gerald Reaven<sup>1,2</sup> presented the theory that insulin resistance played a key role in the aetiology and prognosis of a group of linked metabolic diseases, including, diabetes, hypertension and cardiovascular diseases. He named this group of symptoms 'Syndrome X', which later became known as metabolic syndrome (MetS). This has since been extensively studied in different conditions in the elderly, adults, adolescents and children.<sup>3–6</sup>

In addition, Dr Reaven<sup>2</sup> suggested that insulin resistance presenting compensatory hyperinsulinemia could lead not only to pathogenesis of noninsulin-dependent diabetes mellitus (NIDDM) but also to an increase in the risk of coronary heart disease (CHD).

To corroborate this, he presented an in-depth discussion of the mechanisms involved in Syndrome X, which result in an increased risk of cardiovascular disease (insulin resistance, compensatory hyperinsulinemia, glucose intolerance, increased triglycerides (TGs) and reduced highdensity lipoprotein (HDL) cholesterol concentration). In addition, he extensively investigated insulin resistance syndrome and showed that a large cohort of metabolic abnormalities associated with insulin resistance/hyperinsulinemia can lead to numerous clinical manifestations, including type 2 diabetes, hypertension, cardiovascular disease, polycystic ovary syndrome, non-alcoholic fatty liver disease (NAFLD), certain types of cancer and sleep apnea.7-10 MetS involves a variety of altered metabolic and inflammatory processes, including increases in visceral adiposity. The identification of individuals with this condition is important so that interventions can target lifestyle changes to decrease not only the incidence of diabetes but also the risk of developing cardiovascular disease.<sup>11</sup> Our research team has already started to develop a multicomponent therapy trying to help adolescents with obesity recover their health, taking into account the multifactorial components involved in the aetiology of obesity and its comorbidities.<sup>12</sup> In this respect, we found that they presented high prevalences of insulin resistance (about 70%), dyslipidemias, carotid intima media thickness (cIMT) alterations, hyperleptinemia and hypoadiponectinemia, resulting in an increased prevalence of MetS13,14 with almost 30% of the adolescents having a diagnosis of MetS.<sup>14,15</sup> Interestingly, there was a higher prevalence of NAFLD in adolescents with obesity, reaching 40% and 60% in girls and boys, respectively, aged between 14 and 19 years.<sup>16</sup>

In addition, we were able to show that the most important factors in the aetiology of MetS and NAFLD development in these adolescents with obesity were insulin resistance and visceral adiposity.<sup>14,17</sup> Moreover, de Lima Sanches et al.,<sup>13</sup> showed that an improved insulin resistance index was an independent predictor of cIMT alterations in adolescents with obesity. In addition, Masquio et al.<sup>14</sup> demonstrated that the presence of MetS impaired the reduction in cIMT in adolescents with obesity and that hyperleptinemia correlated with the increased prevalence of NAFLD and the development of atherosclerosis.<sup>18</sup>

However, as far as we are aware, the association between visceral adipose tissue reduction and the impact on insulin resistance and hyperleptinemia, according to the level of reduction, has not been explored in adolescents with obesity analysed after long-term interdisciplinary weight-loss therapy.

## **Methods**

#### Population

This study uses data collected between 2004 and 2012 by the obesity study group. A total of 172 post-pubertal obese adolescents aged 15-19 years of both genders (102 girls and 70 boys) were recruited. The inclusion criteria were as follows: adolescents at Tanner stage five19, the presence of primary obesity and a body mass index (BMI) >95th percentile of the Centers for Disease Control and Prevention (CDC) reference growth charts.<sup>20</sup> Non-inclusion criteria were the use of birth control pills, cortisone, anti-epileptic drugs, a history of renal disease, alcohol intake, smoking and secondary obesity due to endocrine disorders. The study was conducted following the principles of the Declaration of Helsinki and was approved by the Ethics Committee on Research at the Universidade Federal de São Paulo, UNIFESP (152.281), clinical trial Id: NCT01358773. All procedures were explained to those responsible for the volunteers, and a free and informed consent form was signed.

#### Anthropometric measurements

The adolescents were weighed on a scale and after the plethysmograph was used for body composition. (BOD POD equipment - Cosmed, Life Measurement Instruments, Concord, CA, USA), with patients wearing the minimum clothing possible, and height was measured using a stadiometer (Sanny-model ES 2030). BMI was calculated by dividing the weight by height squared (kg/m<sup>2</sup>). Body composition, including fat mass (percentage and kilogrammes) and lean mass (percentage and kilogrammes) was obtained through air displacement plethysmography (BOD POD equipment). Waist circumference was obtained at the midpoint between the last rib and iliac crest.<sup>21</sup>

#### Serum analysis

Blood samples were collected after an overnight fast in the outpatient clinic at approximately 08:00 h. After collection, the blood was centrifuged for 10 min at 5000 r/min and stored at  $-70^{\circ}$ C for future analyses. The materials

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used for collection were disposable and adequately labelled. Blood was collected by a skilled and qualified technician. Insulin resistance was assessed according to the homeostasis model assessment of insulin resistance (HOMA-IR) index. The HOMA-IR was calculated as the product of the fasting blood glucose and the immunoreactive insulin (I) levels: [fasting blood glucose (mg/ dL) × fasting insulin (mU/L)]/405. The quantitative insulin sensitivity check index (QUICKI) was calculated as [1/ (log(fasting insulin) + log(fasting glucose)]. Total cholesterol (TC), TG, HDL, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and hepatic enzymes were analysed using a commercial kit (CELM, Barueri, Brazil). Reference values adopted were as follows: glucose (60-110 mg/dL), insulin (<20 lU/mL), aspartate aminotransferase (AST, 10-40 U/L), alanine aminotransferase (ALT, 10-35 U/L), and c-glutamyl transferase (GGT, 17-30 U/L) as previously described by Sartorio et al.;<sup>22</sup> HOMA-IR (<3.16) according to Keskin et al.;<sup>23</sup> QUICKI (>0.339) according to Schwimmer et al.;<sup>24</sup> TC (<170 mg/ dL), TG (33-129 mg/dL), HDL cholesterol (>38 mg/dL), LDL cholesterol (<130 mg/dL), VLDL cholesterol (10-50 mg/dL) according to Giuliano et al.25

The leptin concentration was measured using a commercially available enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. Leptin values between 1 and 20 ng/mL for males and between 4.9 and 24 ng/mL for females, as previously described by Gutin et al.,<sup>26</sup> were adopted.

The ratios of the lipoprotein levels (TC/HDL and TG/ HDL) were calculated because these ratios have been described in the literature as predictors of cardiovascular disease and MetS in adults and in children.<sup>27–29</sup>

# Visceral and subcutaneous adiposity measurements

The abdominal ultrasonography procedures and the measurements of visceral and subcutaneous fat tissue and fatty liver were performed by the same physician, who was blinded to the subject assignment groups at baseline and at follow-up 1 year after the start of the therapy. This physician was a specialist in imaging diagnostics. A 3.5 MHz multifrequency transducer (broadband) was used to reduce the risk of misclassification. The intra-examination coefficient of the variation for ultrasound (US) was 0.8%. US-determined subcutaneous fat was defined as the distance between the skin and the external face of the rectus abdominal muscle, and visceral fat was defined as the distance between the internal face of the same muscle and the anterior wall of the aorta. The cut-off points for the definition of visceral obesity by ultrasonography were based on the methodology previously described by Ribeiro-Filho et al.30

# Descriptive methodology of interdisciplinary intervention

The interventions were conducted by an interdisciplinary group of health professionals. All adolescents performed the same tests both at the beginning and at the end of the study (Figure 1). Once each month, the adolescents visited the endocrinologist to evaluate the treatment. The adolescents participated in three, 2-h supervised therapy sessions per week (on non-consecutive days) combining physical exercise, physiotherapy, nutritional advice sessions and psychological therapy for 1 year (Figure 1).

*Clinical approach.* All obese adolescents visited the endocrinologist with their parents once every month. The medical follow-up was based on the initial medical history and comprised a physical examination, and the measurement of blood pressure and body mass. The adherence of the adolescents to the interdisciplinary therapies was also assessed. The team discussed some possible changes in lifestyle to promote their health with the adolescents and their parents (Figure 1).

#### Physical exercise practice

Aerobic plus resistance training intervention. During the 1-year therapy period, the adolescents followed a combined physical exercise training therapy. The protocol was performed three times per week for 1 year and included 30 min of aerobic training plus 30 min of resistance training per session. The subjects were instructed to reverse the order of the physical exercises (aerobic and resistance) at each training session. The aerobic training consisted of running on a motor-driven treadmill (Life Fitness - model TR 9700HR) or riding an exercise bicycle at a cardiac frequency intensity representing ventilatory threshold 1 (VT1) (±4 bpm), which was determined by the results of an initial oxygen uptake test for aerobic exercises (ergoespirometry). The physical exercise therapy was based on the guidelines from the American College of Sports Medicine (ACSM).<sup>31</sup> Resistance training was also designed based on ACSM recommendations (Figure 1).32

Nutrition counselling. Energy intake was set at the levels recommended by the Institute of Medicine's dietary reference intake (DRI) for subjects with low levels of physical activity of the same age and gender following a balanced diet.<sup>33</sup> No pharmacotherapies or antioxidants were recommended. Once a week, adolescents had dietetics lessons educating participants on the food pyramid, were taught how to keep diet records and were given information on weight loss diets, fad diets, food labels, dietetics, fat-free foods, low-calorie foods and other related topics. They also had monthly individual consultations (Figure 1).



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Figure 1. Methodological design.

*Physiotherapy intervention*. The adolescents were monitored by a physiotherapist during the therapy in order to prevent musculoskeletal injuries. Once a week, the volunteers had lessons regarding such topics as posture, the prevention of musculoskeletal injuries, diaphragmatic breathing, hydrotherapy, isostretching, and balance.

*Psychological counselling.* Psychological therapy treatment plans were established based on validated questionnaires that considered some of the psychological problems caused by obesity, as described in the literature. These include depression, eating disorders, anxiety, decreased self-esteem and body image disorders. Interdisciplinary therapy consisted of a weekly 1-h group session. Individualized psychological therapy was recommended when it was necessary according to the psychological assessment (Figure 1).

## Statistical analysis

Statistical analysis was performed using the Statistica 12 (StatSoft Inc, Tulsa, USA) software package. The adopted significant value was  $\alpha \leq 5\%$ . Data normality was verified with the Kolmogorov–Smirnov test. Data were expressed as

mean  $\pm$  standard deviation (SD). The effects of 1 year of interdisciplinary therapy were assessed using repeated measures analysis of variance (ANOVA) and Tukey post hoc test. Correlations were established through the Pearson's test. Multivariate regression analysis was performed to look for independent predictors for insulin resistance.

After long-term weight-loss therapy, they were analysed according to the tertile of visceral fat reduction (delta values) – 1st tertile (high): more than 1.8 cm (n=58); 2nd tertile (moderate): between 1.8 cm and 0.79 cm (n=55); and 3rd tertile (low): less than or equal to 0.79 cm (n=59).

# Results

### Baseline condition

Analysing the baseline condition, the adolescents were paired according to BMI and body fat mass as shown in Table 1. This is relevant information that highlights the homogeneity of the sample studied at the initial moment of the interventions. It justifies that the differences that were found between the groups at the final moment are dependent on the effect of the therapy and the group evaluated, according the magnitude of reduction in visceral fat depot.

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	AII		Hign group		Prioderate group		Low group	
	(n = 172)		(n = 58)		(n=55)		(n = 59)	
	Baseline after inter	rvention	Baseline after inte	ervention	Baseline after inte	rvention	Baseline after inte	rvention
Body mass (kg)	100.56 ± 15.01	92.36 ± 15.03 <sup>d</sup>	101.88 ± 14.11	88.22 ± 13.07 <sup>d</sup>	103.34 ± 17.52	95.43 ± 18.03 <sup>d</sup>	96.73 ± 12.80	<b>93.28</b> ± <b>12.86</b> <sup>d</sup>
BMI (kg/m²)	$35.98 \pm 4.59$	$32.91 \pm 4.84^{d}$	$36.06 \pm 4.17$	$30.95 \pm 3.82^{d}$	$36.42 \pm 5.10$	$33.57 \pm 5.34^{d}$	$35.61 \pm 4.51$	$34.29 \pm 4.69^{d}$
Body fat mass (%)	$\textbf{44.09} \pm \textbf{6.97}$	$38.02 \pm \mathbf{8.26^d}$	$44.82 \pm 5.90$	$35.74 \pm \mathbf{7.35^d}$	$43.16 \pm 8.01$	$37.20\pm9.20^{d}$	$\textbf{44.41} \pm \textbf{6.85}$	$41.19 \pm 7.29^{d}$
Lean body mass (%)	$56.00 \pm 6.91$	$61.99\pm8.25^d$	$55.18 \pm 5.90$	$64.26 \pm 7.35^d$	$57.13 \pm 7.80$	$62.80 \pm 9.19^{d}$	$55.60 \pm 6.89$	$58.81 \pm 7.29^{d}$
Body fat mass (kg)	$44.50 \pm 10.97$	$35.60 \pm 11.17^{d}$	$45.88 \pm 10.05$	$31.87 \pm 9.18^{d}$	$44.51 \pm 12.87$	$36.01 \pm 12.86^d$	$43.29 \pm 9.99$	$38.95 \pm 10.28^{d}$
Body lean mass (kg)	$56.02 \pm 9.27$	$56.93 \pm 9.47$	$56.01 \pm 8.41$	$56.79 \pm 9.15$	$58.48 \pm 10.77$	$59.21 \pm 10.83$	$53.60 \pm 7.99$	$54.60 \pm 7.80$
Waist circumference (cm)	$101.55 \pm 10.44$	$94.66 \pm 11.27^{d}$	$103.23 \pm 9.35$	$91.61 \pm 9.81^{d}$	102.21 ± 11.14	$95.20 \pm 11.47^{d}$	$98.87 \pm 10.61$	97.36 ± 11.96
Visceral fat (cm)	$4.41 \pm 1.52$	$3.21 \pm 1.33^{d}$	$5.27 \pm 1.27$	$2.65 \pm 1.06^d$	$4.48 \pm \mathbf{1.54^a}$	$3.22 \pm 1.50^{d}$	$3.52 \pm 1.21_{a,b}$	3.72 ± 1.22℃
Subcutaneous fat (cm)	$3.78 \pm 0.90$	$3.20 \pm 0.91^{d}$	$3.75 \pm 0.84$	$3.09 \pm 0.91^{d}$	$3.85 \pm 1.02$	$3.20 \pm 0.93^{d}$	$3.79 \pm 0.82$	$3.33 \pm 0.85^{d}$
Glucose (mg/dL)	$90.54 \pm 6.54$	$90.34 \pm 7.40$	$90.61 \pm 6.51$	$87.91\pm6.64$	$89.33 \pm 6.83$	91.44 ± 8.06	$91.54\pm6.24$	$91.67 \pm 7.01$
Insulin (uU/mL)	$16.64 \pm 9.22$	$13.12 \pm 8.95^{d}$	$17.05 \pm 9.23$	$11.76\pm7.24^{d}$	$17.07 \pm 11.02$	$14.58 \pm 12.09$	$16.00 \pm 7.29$	I3.2I ± 6.49
HOMA-IR	$3.74 \pm 2.21$	$3.00 \pm 2.37^{d}$	$3.80 \pm 1.97$	$2.58 \pm \mathbf{1.60^d}$	$3.86 \pm 2.87$	3.44 ± 3.38	$3.62 \pm 1.70$	3.03 ± 1.69
QUICKI	$0.32 \pm 0.02$	$0.34 \pm 0.03^{d}$	$0.32 \pm 0.02$	$0.34\pm0.03^{d}$	$0.32\pm0.02$	$0.33 \pm 0.03^{d}$	$0.32 \pm 0.02$	$0.33 \pm 0.02$
Total cholesterol (mg/dL)	$164.89 \pm 33.04$	$155.51 \pm 29.64^{d}$	$170.25 \pm 36.72$	$156.63 \pm 31.14^{d}$	$165.53 \pm 29.77$	$158.65 \pm 31.21^{d}$	$160.00 \pm 31.51$	$151.87 \pm 26.41$
HDL cholesterol (mg/dL)	$45.51 \pm 8.91$	$47.31 \pm 10.02^{d}$	$44.07 \pm 9.49$	$\textbf{45.21} \pm \textbf{9.37}$	$44.11 \pm 8.55$	$46.72 \pm 9.90^{d}$	$48.24 \pm 8.22$	$50.07 \pm 10.42$
LDL cholesterol (mg/dL)	$98.78 \pm 28.68$	$90.55 \pm 25.29^{d}$	$105.26 \pm 33.87$	$94.18 \pm \mathbf{27.96^d}$	$98.98 \pm 22.82$	$94.06 \pm 25.23$	$92.98 \pm 26.95$	$83.75 \pm 21.31^{d}$
VLDL cholesterol (mg/dL)	$20.51 \pm 10.28$	$17.65 \pm 9.27^{d}$	$\textbf{20.84} \pm \textbf{9.58}$	$17.23 \pm 8.69^{d}$	$21.39 \pm 10.36$	$17.87\pm9.28^{d}$	$19.55 \pm 10.92$	$18.05 \pm 9.92$
Triglycerides (mg/dL)	$104.54 \pm 60.59$	$87.97 \pm 46.01^{d}$	$104.19 \pm 48.00$	$86.14 \pm \mathbf{42.86^d}$	$114.40 \pm 76.86$	$89.04 \pm 46.45^{d}$	$96.69 \pm 53.70$	$89.71 \pm 49.24$
TC/HDL	3.74 ± 1.01	$3.41 \pm 0.90^d$	$4 \pm 1.12$	$3.57 \pm 0.91^{d}$	$3.85 \pm 0.89$	$3.52 \pm 0.99^{d}$	$3.41 \pm 0.90$	$3.13\pm0.76^d$
TG/HDL	2.46 ± 1.64	$0.55 \pm 0.23^{d}$	$2.59 \pm 1.55$	$0.55\pm0.26^d$	$2.72 \pm 1.94$	$0.54 \pm 0.19^{d}$	$2.31 \pm 1.38$	$0.57\pm0.24^d$
AST enzyme (U/L)	$24.74 \pm 8.17$	$23.42 \pm 14.27$	$25.07 \pm 8.10$	$21.73\pm\mathbf{6.28^d}$	$24.56 \pm 7.46$	$22.22 \pm 6.17$	$24.26 \pm 8.71$	$26.36 \pm 23.06$
ALT enzyme (U/L)	31.49 ± 18.46	$26.73 \pm 18.37^{d}$	32.77 ± 23.66	$24.59 \pm 14.93$	$30.18 \pm 13.14$	$25.96 \pm 12.85$	31.67 ± 17.24	$29.91 \pm 24.97$
GGT enzyme (U/L)	$25.52 \pm 15.11$	$20.22\pm9.77$ d	$27.93 \pm 22.11$	$20.93 \pm 11.01^{d}$	$25.89 \pm 11.61$	$20.54 \pm 9.55^{d}$	$23.07 \pm 7.55$	19.36 ± 8.68
Leptin (ng/mL)	$37.74 \pm 24.30$	$24.86 \pm 19.83^{d}$	$37.58 \pm 27.10$	$19.03\pm17.24^{d}$	$35.70 \pm 24.25$	$24.93\pm21.75^d$	$40.34 \pm 21.51$	$32.24 \pm 18.80$
BMI: body mass index; HOMA-I	R: homeostasis model	assessment insulin resis	stance; QUICKI: quar	ntitative insulin sensitiv	vity check index; HLD	: high-density lipopro	tein; LDL: low-density	lipoprotein;

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VLDL: very low-density lipoprotein; TC/HDL: total cholesterol/high-density lipoprotein ratio; TG/HDL: triglyceride/high-density lipoprotein ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: c-glutamyl transferase.

Reference values: glucose (60–110 mg/dL), insulin (<201U/mL), AST (10–40U/L), ALT (10–35U/L) and GGT (17–30 U/L) as previously described by Sartorio et al.<sup>32</sup> HOMA-IR (<3.16) according Keskin et al.<sup>32</sup> QUICKI (>0.339) according to Schwimmer et al.<sup>34</sup> total cholesterol (<170 mg/dL), TG (33–129 mg/dL), HDL cholesterol (>130 mg/dL), VLDL cholesterol (<130 mg/dL), VLDL cholesterol (<130 mg/dL), VLDL cholesterol (<10–50 mg/dL) according to Schwimmer et al.<sup>34</sup> total cholesterol (<170 mg/dL), TG (33–129 mg/dL), HDL cholesterol (>138 mg/dL), LDL cholesterol (<130 mg/dL), VLDL cholesterol (<10–50 mg/dL) according to Giuliano et al.<sup>35</sup> Leptin values between 1 and 20 ng/mL for males and between 4.9 and 24 ng/mL for females were adopted as previously described by Gutin et al.<sup>36</sup> Statistical test applied ANOVA-two way post hoc Tukey. <sup>a</sup>Statistical difference of high group in baseline.

<sup>b</sup>Statistical difference of moderate group in baseline condition.

<sup>c</sup>Statistical difference of high group in after intervention.

<sup>3</sup>Statistical difference between values at baseline and after intervention.

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## Effects of interdisciplinary intervention

All sample. Considering the total sample (n=172), there were a statistically significant reduction in body mass (kg), BMI (kg/m<sup>2</sup>), body fat mass (% and kg), waist circumference (cm), visceral fat (cm), subcutaneous fat (cm), insulin ( $\mu$ U/mL), HOMA-IR, TC (mg/dL), LDL cholesterol (mg/dL), VLDL cholesterol (mg/dL), TGs (mg/dL), TC/HDL ratio, TG/HDL ratio, ALT enzyme (U/L), GGT enzyme (U/L) and leptin concentration (ng/mL). On the other hand, there were statistically significant increases in lean body mass (%), QUICKI and HDL cholesterol (mg/dL). For lean body mass (kg), glucose (mg/dL) and AST enzyme (U/L) no statistically significant changes were observed (Table 1).

High group (1st tertile: >1.8 cm). Similarly to the all sample analysis, in the high group, there was a statistically significant reduction in body mass (kg), BMI (kg/m<sup>2</sup>), body fat mass (% and kg), waist circumference (cm), visceral fat (cm), subcutaneous fat (cm), insulin ( $\mu$ U/mL), HOMA-IR, TC (mg/dL), LDL cholesterol (mg/dL), VLDL cholesterol (mg/dL), TGs (mg/dL), TC/HDL ratio, TG/HDL ratio, AST enzyme (U/L), GGT enzyme (U/L) and leptin concentration (ng/mL). In addition, it is important to note that only in this group was the hyperleptinemia state normalized. On the other hand, there was a statistically significant increase in lean body mass (%) and QUICKI. For lean body mass (kg), glucose (mg/dL), HDL cholesterol (mg/ dL) and ALT enzyme (U/L) no statistically significant changes were observed (Table 1).

Moderate group (2nd tertile: between 1.8 and 0.79 cm). In the moderate group, there was a statistically significant reduction in body mass (kg), BMI (kg/m<sup>2</sup>), body fat mass (% and kg), waist circumference (cm), visceral fat (cm), subcutaneous fat (cm), TC (mg/dL), VLDL cholesterol (mg/dL), TGs (mg/dL), TC/HDL ratio, TG/HDL ratio, GGT enzyme (U/L) and leptin concentration (ng/mL). Although, this hormone was not normalized. On the other hand, there was a statistically significant increase in lean body mass (%), QUICKI and HDL cholesterol (mg/dL). For lean body mass (kg), glucose (mg/dL), insulin ( $\mu$ U/ mL), HOMA-IR, LDL cholesterol (mg/dL), AST enzyme (U/L) and ALT enzyme (U/L) no statistically significant changes were observed (Table 1).

Low group (3rd tertile: less than or equal 0.79 cm). In the low group, there was a statistically significant reduction in body mass (kg), BMI (kg/m<sup>2</sup>), body fat mass (% and kg), subcutaneous fat (cm), LDL cholesterol (mg/dL), TC/HDL ratio and TG/HDL ratio. On the other hand, there was a statistically significant increase in lean body mass (%) and visceral fat (cm). For lean body mass (kg), waist circumference (cm), glucose (mg/dL), insulin ( $\mu$ U/mL), HOMA-IR,

QUICKI, TC (mg/dL), HDL cholesterol, VLDL cholesterol (mg/dL), TGs (mg/dL), and AST enzyme (U/L), ALT enzyme (U/L), GGT enzyme (U/L) and leptin concentration (ng/mL) no statistically significant changes were observed (Table 1).

Analysing the delta values. According to the delta values analysed, it is possible to note a greater effectiveness of high group than the moderate and low groups in promoting a reduction in body mass (kg), body fat mass (% and kg), visceral fat (cm) and leptin concentration (ng/mL). Interestingly, the programme was more effective in the high group in respect of improved BMI (kg/m<sup>2</sup>) and QUICKI than in the moderate group. For the variables lean body mass (kg), waist circumference (cm), subcutaneous fat (cm), glucose (mg/dL), insulin ( $\mu$ U/mL), HOMA-IR, TC (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), TGs (mg/dL), TC/HDL ratio, TG/HDL ratio, AST, ALT and GGT enzymes no significant differences were observed (Table 2).

*Multivariate logistic regression analysis.* In the regression analysis of the total sample, both visceral fat and BMI were predictors of increased insulin resistance. Moreover, for the high and moderate groups, the regression model demonstrated that only visceral fat was an independent predictor of an increase in the insulin resistance index (HOMA-IR). Considering the low group, only BMI (kg/m<sup>2</sup>) was an independent predictor of an increase in the insulin resistance in the insulin resistance index (HOMA-IR) (Table 3).

Correlations analysis. Performing the correlation analysis, as demonstrated in Figure 2(a) to (d), visceral fat (cm) was positively correlated with glucose (mg/dL): r=0.22, p=0.001; insulin ( $\mu$ U/L): r=0.38, p=0.0001 and HOMA-IR: r=0.40, p=0.001. Moreover, negative correlations were found between visceral fat and QUICKI: r=-0.38, p=0.0001. Considering the lipid profile, as shown in Figure 3(a) to (d), there were positive correlations between visceral fat (cm) and TC (mg/dL): r=0.23, p=0.006; LDL cholesterol (mg/dL): r=0.22, p=0.01; VLDL cholesterol (mg/dL): r=0.26, p=0.002 and TGs (mg/dL): r=0.26, p=0.002. Regarding the hepatics enzymes, as shown in Figure 4(a) to (d), there were positive correlations between visceral fat (cm) and AST enzyme (U/L): r=0.35, p=0.0001; ALT enzyme (U/L): r=0.37, p=0.0001 and GGT enzyme (U/L): r=0.46, p=0.0001.

# Discussion

This study aimed to investigate the impact of visceral adipose tissue reductions, analysed in terms of their magnitude, on insulin resistance and hyperleptinemia in a sample of adolescents with obesity. Interestingly, the most important finding was that a greater reduction in visceral adipose

	All	high group	moderate group	low group
	(n=172)	(n=58)	(n=55)	(n = 59)
Body mass (kg)	$-11.87 \pm 21.88$	$-18.30 \pm 22.04$	$-7.76 \pm 20.73^{a}$	$-9.64 \pm 21.89^{a}$
BMI (kg/m <sup>2</sup> )	<b>−4.41</b> ± 7.96	-6.74 ± 7.56	$-2.79 \pm 7.75^{a}$	$-3.72 \pm 8.18$
Body fat mass (%)	$-7.34 \pm 9.73$	$-10.96 \pm 8.56$	$-5.85\pm9.78^{\mathrm{a}}$	$-5.32\pm9.96^{\mathtt{a}}$
Lean body mass (%)	$\textbf{4.08} \pm \textbf{14.91}$	$\textbf{5.70} \pm \textbf{15.93}$	6.61 ± 14.18	$0.13\pm14.08^{\rm a}$
Body fat mass (kg)	-9.76±11.54	$-15.69 \pm 9.62$	$-7.54 \pm 12.87^{a}$	$-6.20\pm9.77^{\rm a}$
Body lean mass (kg)	$-0.74 \pm 14.60$	-2.22 ± 17.67	$1.78 \pm 14.03$	$-1.68 \pm 11.52$
Waist circumference (cm)	-12.81 ± 58.92	$-21.24 \pm 57.50$	-12.91 ± 50.58	$-4.42 \pm 67.22$
Visceral fat (cm)	-1.21 ± 1.36	$\textbf{-2.63}\pm\textbf{0.82}$	$-1.26 \pm 0.28^{a}$	$0.20\pm0.83^{\rm a,b}$
Subcutaneous fat (cm)	$-0.62\pm0.81$	$-0.66\pm0.78$	$-0.73 \pm 0.89$	$-0.47\pm0.74$
Glucose (mg/dL)	-2.84±17.21	$-4.25 \pm 13.89$	$\textbf{0.45} \pm \textbf{14.78}$	$-4.62 \pm 21.71$
Insulin (uU/mL)	-4.01 ± 9.71	$-5.50 \pm 9.15$	$-2.75 \pm 12.60$	$-3.76 \pm 6.70$
HOMA-IR	$-0.86 \pm 2.47$	$-1.27\pm2.03$	$-0.48 \pm 3.45$	$-0.82\pm1.60$
QUICKI	$\textbf{0.00} \pm \textbf{0.07}$	$\textbf{0.02}\pm\textbf{0.05}$	$0.01\pm0.06^{\mathrm{a}}$	$-0.01\pm0.09$
Total cholesterol (mg/dL)	-13.91 ± 33.53	$-16.37 \pm 31.45$	-9.76 ± 24.78	$-15.86 \pm 41.98$
HDL cholesterol (mg/dL)	$\textbf{0.45} \pm \textbf{10.04}$	$\textbf{0.35} \pm \textbf{9.30}$	$1.76\pm7.93$	$-0.71 \pm 12.38$
LDL cholesterol (mg/dL)	$-10.12 \pm 24.95$	$-12.74 \pm 23.16$	$-4.84 \pm 24.67$	$-12.95 \pm 26.50$
VLDL cholesterol (mg/dL)	$-3.08\pm9.81$	<b>−</b> 3.91 ± 7.58	-3.45±11.18	-1.91 ± 10.47
Triglycerides (mg/dL)	$-19.07 \pm 50.40$	$-19.56 \pm 37.87$	$-26.98 \pm 60.26$	$-11.27 \pm 50.99$
TC/HDL	$-0.33\pm0.53$	$\textbf{-0.43} \pm \textbf{0.52}$	$-0.33 \pm 0.58$	$-0.24\pm0.46$
TG/HDL	-1.90 ± 1.53	$-2.03\pm1.39$	−2.1 ± 1.84	$-150, \pm 1.26$
AST enzyme	-1.86 ± 15.07	$-3.72 \pm 7.24$	-2.75 ± 7.32	$-8.61 \pm 23.53$
ALT enzyme	<b>−</b> 5.4 ± 18.95	-8.61 ± 17.56	-4.69±10.18	$-3.00\pm25.57$
GGT enzyme	$-5.82\pm10.89$	-7.37±15.17	$-5.73 \pm 8.04$	$-4.46 \pm 7.76$
Leptin (ng/mL)	$-8.62\pm22.80$	$-15.62 \pm 25.56$	$-9.28\pm19.47^{\rm a}$	$0.83\pm19.65^{\rm a}$

**Table 2.** Comparison of delta values among visceral fat groups.

BMI: body mass index; HOMA-IR: homeostasis model assessment insulin resistance; QUICKI: quantitative insulin sensitivity check index; HLD: highdensity lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; TC/HDL: total cholesterol/high-density lipoprotein ratio; TG/ HDL: triglycerides/high-density lipoprotein ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: c-glutamyl transferase. Statistical test applied ANOVA-one way post hoc Tukey.

<sup>a</sup>Statistical difference compared to high group.

<sup>b</sup>Statistical difference compared to moderate group.

Table 3.	Multivariate	logistic re	gression ana	lysis of	f association	between	insulin	resistance and	l bod	y com	position	parameters.

Variables	95% CI			p value
	OR	Lower	Upper	
Total sample				
Visceral fat (cm)	1.604	1.303	1.975	0.0001
Lean body mass (%)	0.698	0.003	146.86	0.895
Body fat mass (%)	0.692	0.003	147.28	0.893
BMI (kg/m <sup>2</sup> )	1.149	1.056	1.249	0.001
High group				
Visceral fat (cm)	1.876	1.246	2.798	0.002
Lean body mass (%)	0.001	10-11	104.243	0.423
Body fat mass (%)	0.001	10-11	147.86	0.422
BMI (kg/m <sup>2</sup> )	1.07	0.922	1.241	0.375
Moderate group				
Visceral fat (cm)	2.567	1.676	3.931	0.0001
Lean body mass (%)	1.03	0.934	1.135	0.55

(Continued)

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#### Campos et al.

#### Table 3. (Continued)

Variables	95% CI			þ value
	OR	Lower	Upper	
Body fat mass (%)	I	0.989	1.354	_
BMI (kg/m <sup>2</sup> )	1.157	0.922	_	0.069
Low group				
Visceral fat (cm)	1.068	0.745	1.53	0.722
Lean body mass (%)	1.324	0.014	126.81	0.904
Body fat mass (%)	1.321	0.013	130.27	0.905
BMI (kg/m <sup>2</sup> )	1.207	1.072	1.36	0.002

Statistical significance is bold in the Table. BMI: body mass index; CI: confidence interval; OR: odds ratio; HOMA-IR: homeostasis model assessment insulin resistance. Multivariate logistic regression analysis was realized considering HOMA-IR as a dependent variable. The analysis was performed in different conditions: all sample; high group, moderate group and low group.

BMI: body mass index; CI: confidence interval; OR: odds ratio; HOMA-IR: homeostasis model assessment insulin resistance.

Model considering HOMA-IR as a dependent variable. All sample (n = 172). BMI: body mass index (kg/m<sup>2</sup>).

Model considering HOMA-IR as a dependent variable. Only high group considered in the analysis. BMI: body mass index (kg/m<sup>2</sup>).

Model considering HOMA-IR as a dependent variable. Only moderate group considered in the analysis. BMI: body mass index (kg/m<sup>2</sup>). Model considering HOMA-IR as a dependent variable. Only low group considered in the analysis. BMI: body mass index (kg/m<sup>2</sup>).



**Figure 2.** Correlations of visceral fat with: (a) glucose (mg/dL): r = 0.22, p = 0.001; (b) insulin ( $\mu$ U/L): r = 0.38, p = 0.0001; (c) HOMA-IR: r = 0.40, p = 0.001 and (d) QUICKI: r = -0.38, p = 0.0001.

tissue (>1.8 cm; according to the delta value analysis) was a cut-off point to obtain a significant decrease in insulin resistance and normalization of leptin concentration (Table 1).

Corroborating this, the multivariate logistic analysis showed that increased visceral fat is an independent predictor of increased insulin resistance in the groups with a high and moderate reduction of adipose deposits (Table 3). In a previous investigation, it was shown that improved HOMA-IR was an independent predictor of cIMT alterations in a sample of adolescents with obesity.<sup>13</sup> In fact, in

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**Figure 3.** Correlations of visceral fat with: (a) total cholesterol (mg/dL): r = 0.23, p = 0.006; (b) LDL cholesterol (mg/dL): r = 0.22, p = 0.01; (c) VLDL cholesterol (mg/dL): r = 0.26, p = 0.002 and (d) triglycerides (mg/dL): r = 0.26, p = 0.002.



**Figure 4.** Correlations of visceral fat with: (a) AST enzyme (U/L): r = 0.35, p = 0.0001; (b) ALT enzyme (U/L): r = 0.37, p = 0.0001 and (c) GGT enzyme (U/L): r = 0.46, p = 0.0001.

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this investigation, there was only a significant decrease in insulin resistance and insulin concentration in the group which presented a higher reduction in visceral fat after long-term interdisciplinary weight-loss therapy occurs.

Remarkably, our data indicate that when adolescents with obesity have a high or moderate decrease in the visceral adipose tissue, there are improvements in 20 and 16 analysed variables, respectively, including body composition and glucose and lipid metabolism. However, when there was only a small reduction in visceral adipose tissue, there were only improvements in nine parameters (Table 1).

These results show that reduced visceral adipose tissue after long-term weight-loss therapy is a key factor in the control of insulin resistance and a range of metabolic conditions, looking at a sample of obese adolescents with similar body mass, BMI, body fat and subcutaneous fat at baseline, as shown in Table 1. This strongly corroborates the thesis of Dr Reaven<sup>34–36</sup> regarding insulin resistance as a key aspect in the development of many chronic diseases. Our research suggests that if these conditions are not treated early on in obese adolescents, they may develop not only insulin resistance but also MetS,<sup>15</sup> NAFLD,<sup>37</sup> sleep apnea,<sup>38</sup> bone metabolism<sup>39</sup> and cardiovascular disease.<sup>13</sup>

The present study showed the key role of visceral fat in the modulation of metabolic and hormonal conditions in a sample of adolescents with obesity, including positive correlations with TC, LDL cholesterol, VLDL cholesterol, TGs, glucose, insulin, HOMA-IR and hepatic enzymes. Moreover, negative correlations between visceral fat were observed with the insulin sensitivity index (QUICKI) (Figures 2 to 4). The data demonstrate the intrinsic link between high visceral fat and insulin resistance in adolescents with obesity, which may lead to the development of metabolic alterations and a consequent increase in cardiovascular risk in this population.<sup>13,17,40</sup>

In addition, it is important to note that leptin levels, a potent biomarker of energy balance and inflammatory processes related to obesity, were significantly lower after both high and moderate reductions in visceral adipose tissue. In the group presenting a high reduction of visceral fat, the leptin values reached normal concentrations, while the adolescents with a moderate reduction continued to present hyperleptinemia. In agreement with our hypothesis, the group presenting a low delta reduction in deposits showed no significant change in leptin concentration (Table 1).

Importantly, hyperleptinemia can impair energy balance, specifically in its anorexigenic effects upregulating  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH).<sup>41</sup> Indeed, hyperleptinemia can weaken the anti-inflammatory effects of adiponectin, the key hormone involved in the modulation of glucose and lipid metabolism in insulin-sensitive tissues.<sup>42</sup>

In summary, the metabolic and inflammatory parameters of the adolescents who lost a high proportion of visceral adipose tissue (>1.8 cm) improved, while they did not in those who had a small reduction (<0.79 cm). Therefore, long-term interdisciplinary therapy was shown to contribute to the control of related comorbidities.

In conclusion, the present study showed that the magnitude of reduction in visceral fat was an independent predictor for insulin resistance control, hyperleptinemia and a range of altered metabolic conditions observed in obese adolescents.

#### **Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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#### **ORCID iD**

Raquel Munhoz da Silveira Campos D https://orcid.org/0000 -0001-6132-4349

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# **Personal Memories**

Diabetes & Vascular Disease Research 2019, Vol. 16(2) 207–209 © The Author(s) 2019 Article reuse guidelines: sagepub.com/journals-permissions DOI: 10.1177/1479164119829999 journals.sagepub.com/home/dvr SAGE

Jerry loved the Cleveland Indians baseball team and, even though they would always break his heart, he remained loyal. During baseball season if you wandered by his office, you could often hear the games streaming over the Internet

Joshua W. Knowles, MD, PhD, FAHA, FACC Assistant Professor of Medicine Division of Cardiovascular Medicine Stanford University

Jerry, you showed me how to be passionately dispassionate about what is revealed from real data, about how ideas can evolve and sometimes turn on a dime, and how intellectual honesty and integrity are the foundation. You live on in us.

PS And I forgive you for the brain damage you gave me in doing the first AACE Consensus Conference on the Insulin Resistance Syndrome so many years ago!

Dan Einhorn, MD, FACP, FACE Chief, Diabetes and Endocrine Associates Medical Director, Scripps Whittier Diabetes Institute Clinical Professor, UCSD School of Medicine La Jolla, California

As an endocrine fellow and junior faculty member, Dr Reaven was always the Father of Insulin Resistance. Through kind words, encouragement and advice, he was important in my career development as he was for many other physician scientists. I remember him warmly and wish there were more like him.

W. Timothy Garvey, MD Butterworth Professor and Chair Department of Nutrition Sciences GRECC Investigator and staff physician Birmingham VA Medical Center

Was 2 years into my endocrine practice in 1988 when Dr Gerald Reaven described Syndrome X, coincident with the descriptions of the Atherogenic Pattern B Dyslipidemia Phenotype, and the introduction of the ATP I Cholesterol Guidelines. As a clinician, this coalescence gave me explanations to communicate with patients, with and without diabetes, and directions, with the tools available at the time, and the drive to learn more, to reduce their risk for atherosclerosis burden. Four years later I was honoured that he accepted my invitation to be our Keynote Speaker for our Annual Orange County, CA, Chapter, ADA, Diabetes Management Symposium. So immense his contribution, so famous had he become, yet so humbly he accepted a distinguished research clinician plaque as we honoured him.

Paul D. Rosenblit MD, PhD, FACE, FNLA Clinical Professor, Medicine (Div. Endocrinology, Diabetes, Metabolism), University California, Irvine (UCI), School of Medicine,

Irvine, CA Many years ago, I was a young electrophysiologist inter-

ested in ventricular arrhythmias and Dr Reaven showed me cellular pathways with free fatty acids and electrical disturbances. It was amazing how it controlled life-threatening arrhythmia with use of insulin in an extremely hightriglyceride patient having a myocardial infarction. I will never forget that.

Robert J. Chilton, DO, FACC Professor of Medicine The University of Texas San Antonio, Texas

I first met Jerry Reaven when I was a graduate student in the laboratories of Dr Bernard Jeanrenaud and Dr Albert E Renold in Geneva in the late 1970s. He paid many visits there at our Institut de Biochimie Clinique and also did a sabbatical. He was always eager to meet students and I had many lively and inspiring scientific discussions with him and his wife Eve. I was struck by his enthusiasm and drive and could see the joy of doing science in his eyes, and all this was very inspiring for someone starting his career in medical research. It was so moving for me to give a lecture in his memory after all these years at the last WCIRDC meeting.

Very few scientists can claim that they have identified a disease that is, furthermore, of high prevalence in our modern societies. But this applies to Jerry Reaven. Metabolic syndrome is now listed as a disease entity (E88.81) in the International Classification of Diseases-10, avowing to the outstanding contribution of Jerry Reaven in bringing this clustering of factors involved in cardiometabolic diseases to the attention of clinicians and scientists.

#### Diabetes & Vascular Disease Research 16(2)

Marc Prentki, PhD Professor of Nutrition, Biochemistry and Molecular Medicine, Université de Montréal, Montreal, Canada

In addition to being a renowned scientist, Jerry was an ardent baseball fan, particularly of the Cleveland Indians. He and I often celebrated our July birthdays by watching the Indians play. It was always fun to watch him score baseball the 'old fashion way' by scorekeeping on a printed baseball scorecard and hear him relay stories of watching Bob Feller play. He also always refused to apply sunscreen.

Sun H. Kim, MD, MS Associate Professor of Medicine Stanford University School of Medicine, California

At 8:00 am on 31 Aug 2016, I approached the lecture to a Grand Rounds lecture at Stanford School of Medicine. Heads went up as my first slide, entitled Insulin Resistance, Obesity and Metabolic Syndrome, flickered on. With Gerry Reaven sitting in the front row I began by saying, 'Despite conventional wisdom, anyone who thinks they understand insulin resistance is simply wrong'. Following the lecture, I had the opportunity to visit with Gerry and was struck by a very cluttered office, with a dusty desk on which was scattered a half-dozen or so, highly oxidised medallions, including the 1988 Banting Medal. Looking back, I carry with me the clear impression of a man animated by love of science and not accolades. A role model for us all. With warmth and affection,

Ronald M. Evans, PhD Howard Hughes Medical Institute Salk Institute for Biological Studies La Jolla, California

Dr Reaven: the giant in research with the gentle touch. Always willing to hear you and let you know whether in his opinion you were right or wrong, and when wrong, letting you know in such a way that it never hurt you. What a quality of a man!

Jaime A. Davidson, MD, FACP, MACE Clinical Professor of Medicine The University of Texas Southwestern Medical Center Dallas, Texas

In 1978, along with Don Chisholm from Sydney, we published a paper in Diabetologia – 'The relationship of insulin response to a glucose stimulus over a wide range of glucose tolerance'. This demonstrated an inverted U-shaped pattern that is now known as 'The Starlings Curve of the Pancreas'. We believed we were the first to describe this. Within days, I received a polite but pointed note from a gentleman called Gerry Reaven from Stanford. He pointed out that he had reported this phenomenon 10 years earlier! Swallowing my pride, I made contact, and several months later while visiting the United States, visited and met Gerry in Palo Alto over dinner, for the first time. There we settled our 'differences' on the matter of publication priority. As to the key driving force/s of the Metabolic Syndrome, which he called Syndrome X, we differed on this issue, but it was the start of a friendship that spanned almost three decades.

Paul Zimmet AO, PhD, FRACP, FRCP, FTSE Professor of Diabetes Research, Monash University, Melbourne, Australia

Dr Gerald Reaven was the penultimate clinical investigator with unquestioned integrity, intellectual curiosity and creativity. He served as a role model for multiple generations of young investigators and I am proud to claim that he served as my role model.

Ralph A. DeFronzo, MD Professor of Medicine Chief, Diabetes Division University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, Texas

Jerry told me that before the Banting lecture, another research had turned to him and said, 'Jerry, no one remembers if you are good but you will be remembered forever if you are bad'. To which Jerry replied 'Here is to my infamy'.

Sue Kim, MD, MS Associate Professor of Medicine Stanford University School of Medicine California

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Gerald Reaven was a giant in his field, who positively influenced the careers of countless individuals, including myself. Possessed of an intellectual, inquiring mind and amazing drive and energy, he made a contribution that can truly be said to have changed medicine. In fact the journal hosting this special edition celebrating Dr Reaven's life came into existence because of his work. I only really got to know Gerald in the last 15– 20 years of his life and I discovered that the self same man who scared the life out of me with his penetrating questions in Denmark in the late 1980s was also extremely generous and kind as well as a great scientist – what a life!

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#### Others

Peter J Grant, FMed Sci Professor of Medicine University of Leeds, Leeds UK

After travelling on some of the same circuits a number of years ago, Jerry and I realised, more or less simultaneously, that the atherogenic dyslipidemia that I had been working on for some time looked very much like the lipid component of his 'Syndrome X'. So, sparked in large part by his characteristic energy and enthusiasm, we embarked on a collaboration that clearly linked these traits, and demonstrated Jerry's unerring ability to strike at the heart of a question, and to nail the answer with elegance and simplicity.

Ronald M. Krauss, MD Senior Scientist and Director, Atherosclerosis Research, Children's Hospital Oakland Research Institute,

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Clinical Chemistry Point/Counterpoint

California

Point "Metabolic syndrome: Requiescat in pace" by Jerry Counterpoint "Metabolic syndrome: Still Lives" by Grundy Response "Metabolic syndrome" Just being alive is not enough, for as Sportin' Life points out in *Gershwin's Porgy and Bess*, "Methuselah lived 900 years, but who calls it living, when no gal will give in to him." by Jerry

Sun H Kim, Associate Professor of Medicine Stanford University School of Medicine, California



Gerald Reaven (right) and Yehuda Handelsman at the World Congress of Insulin Resistance, Los Angeles, California



Gerald Reaven (left) receives an award from Yehuda Handelsman at the World Congress of Insulin Resistance, Los Angeles, California

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