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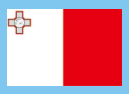

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EDITORIAL

Excess vitamin intake: An unrecognized risk factor for obesity

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Abstract

Over the past few decades, food fortification and infant formula supplementation with high levels of vitamins have led to a sharp increase in vitamin intake among infants, children and adults. This is followed by a sharp increase in the prevalence of obesity and related diseases, with significant disparities among countries and different groups within a country. It has long been known that B vitamins at doses below their toxicity threshold strongly promote body fat gain. Studies have demonstrated that formulas, which have very high levels of vitamins, significantly promote infant weight gain, especially fat mass gain, a known risk factor for children developing obesity. Furthermore, ecological studies have shown that increased B vitamin consumption is strongly correlated with the prevalence of obesity and diabetes. We therefore hypothesize that excess vitamins may play a causal role in the increased prevalence of obesity. This review will discuss: (1) the causes of increased vitamin intake; (2) the non-monotonic effect of excess vitamin intake on weight and fat gain; and (3) the role of vitamin fortification in obesity disparities among countries and different groups within a country.

Key words: Vitamin fortification; Refined grain; Infant formula; Obesity; Diabetes; Insulin resistance; Oxidative stress; Glycemic index; Formula feeding; Epigenetic

Core tip: B vitamins are a known fat gain promoting factor. Food fortification-induced high vitamin consumption is followed by a rapid increase in obesity prevalence. Why is the fat gain effect of B vitamins neglected in obesity studies? Why does obesity prevalence vary from country to country? Why are the poor in developed countries but the rich in developing countries at high risk of obesity? Why is obesity prevalence higher in blacks than whites in the United States? Why does formula feeding (which is associated with high energy expenditure) increase the risk for obesity? Why is physical inactivity associated with increased obesity risk? This paper reviews the role of excess vitamins in obesity and proposes a unified answer to these questions.

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INTRODUCTION

Obesity, a state of excessive accumulation of fat in the body, is a major risk factor for many diseases, such as type 2 diabetes and cardiovascular disease[1,2]. In the 1970s and 1980s, a rapid increase in the prevalence of obesity occurred almost simultaneously in many developed countries. Since then, developing countries have also experienced a rapid increase in obesity rates[3,4]. Nowadays, obesity has become a global epidemic[5]. It is worth noting that the prevalence of obesity differs greatly among

Table 1 The estimated daily average requirements and recommended dietary allowances for selected vitamins (mg/d)[1]

Vitamin	Adult man		Adult woman		Pregnancy	
	EAR	RDA	EAR	RDA	EAR	RDA
Thiamin	1.0	1.2	0.9	1.1	1.2	1.4
Riboflavin	1.1	1.3	0.9	1.1	1.2	1.4
Niacin	12	16	11	14	14	18
Vitamin B_6	1.1	1.3	1.1	1.3	1.6	1.9
Vitamin C	75	90	60	75	70	90
Vitamin E	12	15	12	15	12	15

[1]Data are from the United States Food and Nutrition Board. EAR: Estimated daily average requirement, available from: URL: http://iom.edu/Activities/Nutrition/SummaryDRIs/~/media/Files/Activity%20Files/Nutrition/DRIs/EAR%20Table.pdf. RDA: Recommended dietary allowance, available from: URL: http://iom.edu/Activities/Nutrition/SummaryDRIs/~/media/Files/Activity%20Files/Nutrition/DRIs/RDA%20and%20AIs_Vitamin%20and%20Elements.pdf.

countries[3,4,6,7] as well as groups within a country[8-12]. It is more prevalent among those with low socioeconomic status (SES) in developed countries[6,8-10] but with high SES in developing countries, especially at their early stage of development[10-12]. Interestingly, compared with breast-fed infants, formula-fed infants have higher rather than lower levels of energy expenditure[13,14] and are more at risk for obesity in later life[15-17]. Therefore, the rapidly increased prevalence of obesity cannot be simply explained by genetic factors or decreased energy expenditure.

Recently, it has been suggested that changes in the global food system may play a role in the increased prevalence of obesity[4]. If this is the case, the global food system must have sharply changed in the 1970s-1980s. Notably, in the 1970s and 1980s, the contents of vitamins (organic chemicals affecting the body's functioning) in the food system of many developed countries were sharply increased due to modifications or changes in their rules, laws and regulations regarding food fortification[18-20]. This led to a nationwide increase in the consumption of many vitamins, especially fat synthesis-promoting B vitamins[21-24], including B_1 (thiamin), B_2 (riboflavin), B_3 (niacin) and B_6, in many countries[18-20]. Thus, there is a possibility that the food fortification-induced high vitamin intake may be related to the sudden increase in the prevalence of obesity in the 1970s-1980s. Indeed, emerging evidence suggests that this food fortification-induced excess vitamin intake might play a major role in the increased prevalence of obesity[25,26]. In this review, we will discuss the cause of increased vitamin intake and its possible role in obesity, as well as the obesity disparities among countries and groups within countries.

CAUSES OF EXCESS VITAMIN INTAKE

Until the mid 1930s when the first commercial yeast extract vitamin B complex and semi-synthetic vitamin C supplement tablets were sold, vitamins were obtained solely through natural foods and seasonal changes in diet usually greatly altered the types and amounts of vitamins ingested. For example, the intake of fresh vegetable-derived vitamins might be high in summer but low in winter. However, through evolution, humans have adapted to this seasonal variations in vitamin intake by developing mechanisms to maintain the vitamin homeostasis. While the intake of vitamins is higher in summer, their elimination through sweat and sebum[27-30] may also increase because the secretion of sweat and sebum is higher in summer than in winter[28,31,32]. Moreover, the body can store a certain amount of vitamins when the supply is adequate, which can be used for some time when the intake is inadequate. For example, it will take several months before the first symptoms of vitamin C deficiency appear in a vitamin C deprivation condition[33]. From this point of view, it seems unnecessary to take vitamins everyday, although estimated daily average requirements (EARs) and the recommended dietary allowances are given (Table 1). Yet over the past several decades, the actual intake of vitamins has been significantly higher than the EARs due to the following causes.

Increased vitamin intake from vegetable/fruit sources

Over the past several decades, many fresh vegetables and fruits with better quality can be obtained year round due to widespread out-of-season cultivation. This has not only led to an increase in the intake of vegetable/fruit-derived vitamins (*e.g.*, vitamin C), but also abolished the seasonal vitamin intake variations. Taking the United States as an example, the per capita consumption of vegetables and fruits showed an increasing trend in the 1970s through the 1990s (Figure 1A), leading to an increase in vitamin C intake since the mid-1960s[34].

Increased vitamin intake from animal sources

The consumption of animal-based foods significantly increased in developed countries in the second half of the last century. Dietary patterns in developing countries have been shifting to a more meat-centric diet over the past few decades[5,35]. Such a nutrition transition has increased the intake of vitamins (especially nicotinamide, a form of niacin) from animal-based foods. For example, United States per capita consumption of total meat showed an increasing trend between the 1930s and 2000 (Figure 1B), which increased the daily intake of meat-derived nicotinamide from 6.8 mg in the 1930s to 11.4 mg in 2000, according to the data on meat contribution to daily niacin intake[34].

Increased vitamin intake from artificial sources

Besides increased natural vitamin sources, vitamins may be obtained from artificial sources, which involves food fortification, infant formula fortification, and vitamin-enriched drinks. Fortification is the process of adding synthetic vitamins to foods and infant milk (including breast milk or formula) to increase its overall vitamin content[34]. Some staple foods (such as flour and maize) are used as a vehicle for fortification. Wheat flour fortification with synthetic vitamins (B_1, B_2 and niacin) was started first in the United States in the late 1930s, which was soon adopted by many developed countries and then introduced

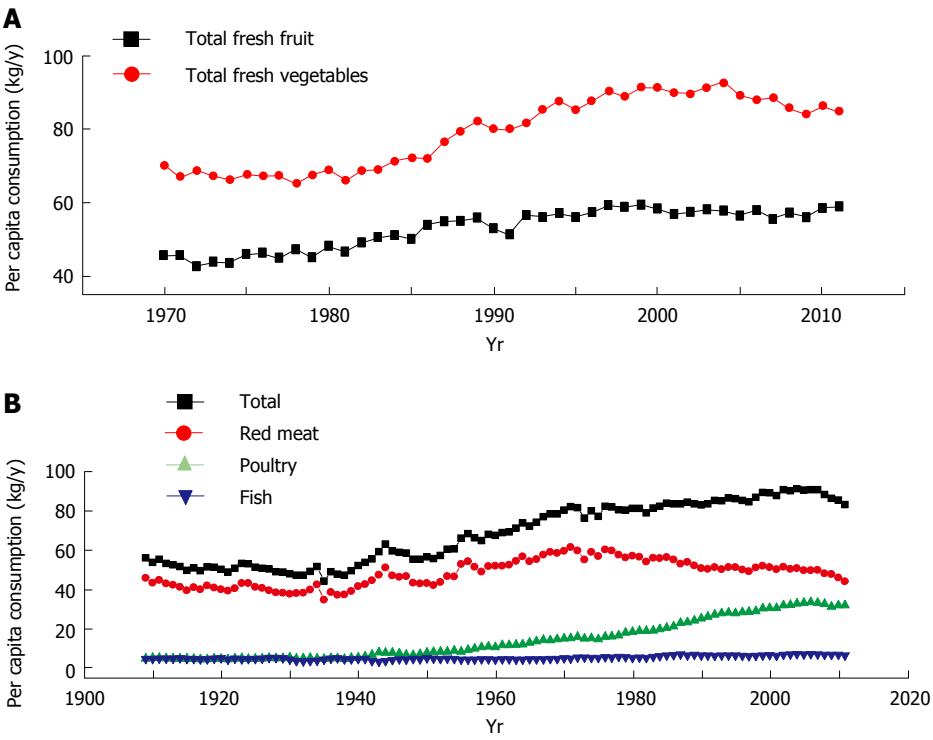

Figure 1 Trends in United States per capita consumption of vegetables, fruits (A) and meats (B). Data are from the Economic Research Service of the United States Department of Agriculture. Available from: URL: http://www.ers.usda.gov/data-products/food-availability-(per-capita)-data-system/.aspx.

Table 2 Fortification recommendations for ready-to-eat cereals

Vitamin	U.S. RDA (mg/d)	1974-1992 amount (mg/per pound)[1]	1974-2000 amount (mg/per pound)[1]
Thiamin	1.5	6	5.7
Niacin	20	80	76
Riboflavin	1.7	6.8	6.4
Vitamin C	60	240	227
Vitamin B_6	2	8	7.6

[1]Data are from Reference 34. RDA: Recommended dietary allowance.

to developing countries[19,20]. Notably, ready-to-eat cereals are a major vehicle of fortification of B vitamins (B_1, B_2, B_6 and niacin). Especially since 1974 when the food fortification standards for cereals were updated, ready-to-eat cereals have become the top food source of many vitamins[20,34]. The levels of vitamins in fortified ready-to-eat cereals are so high (Table 2) that consumption of less than a quarter pound of them (because foods *per se* also contain some amount of vitamins) meets the daily need for these vitamins in an adult. Many sugar-sweetened beverages are also supplemented with vitamins[36,37], which is also an important cause of increased vitamin intake. Since the 1950s, synthetic vitamins have been added to infant formulas[38]. In the 1980s, the governments of most countries established minimum nutrient requirements for commercial infant formulas[39], resulting in a significant increase in the content of vitamins in formulas. The levels of vitamins in some formulas for premature infants are more than 20 times higher than that of human milk (*i.e.*, about the minimum limit for nutrients) (Table 3). This leads to a high vitamin intake in infancy.

As a result of the combination of the above factors, the intake of vitamins has been significantly increased

Table 3 The minimum limit for infant formulas in the United States and commercially labeled values of nutrients (per 100 kcal)

Nutrient	ML[1]	TF[2]	TF/ML	PF[2]	PF/ML
Macronutrients					
Protein (g)	1.8	2.71	1.5	3	1.7
Fat (g)	3.3	5.27	1.6	5.43	1.7
Vitamins					
Vitamin B_1 (μg)	40	100	2.5	250	6.3
Vitamin B_2 (μg)	60	150	2.5	620	10.3
Niacin (nicotinamide, μg)	250	1050	4.2	5000	20.0
Vitamin B_6 (μg)	35	60	1.7	250	7.1
Vitamin B_{12} (μg)	0.15	0.25	1.7	0.55	3.7
Vitamin C (mg)	8	9	1.1	37	4.6
Biotin (μg)	1.5	4.4	2.9	37	24.7
Pantothenic acid (mg)	300	450	1.5	1900	6.3
Folic acid (μg)	4	15	3.8	37	9.3
Vitamin A (IU)	250	300	1.2	1250	5.0
Vitamin D (IU)	40	60	1.5	150	3.8
Vitamin E (IU)	0.7	1.5	2.1	4	5.7
Vitamin K (μg)	4	8	2.0	12	3.0

[1]The minimum limit for nutrients set by the United States Infant Formula Act of 1980[40]; [2]Similac formulas (http://abbottnutrition.com/brands/similac). ML: Minimum limit; TF: A similac formula for term infants (Similac Expert Care® 24 Cal With Iron); PF: A Similac formula for low-birth-weight infants and premature infants (Similac® Special Care® 20 With Iron).

over the past few decades. As shown in Figure 2, United States per capita daily consumption of vitamin B_1, B_2 and niacin has doubled from the 1930s to 2000, which is significantly higher than the EARs.

FOOD FORTIFICATION-RELATED DISPARITIES

Food fortification may lead to differential exposure to

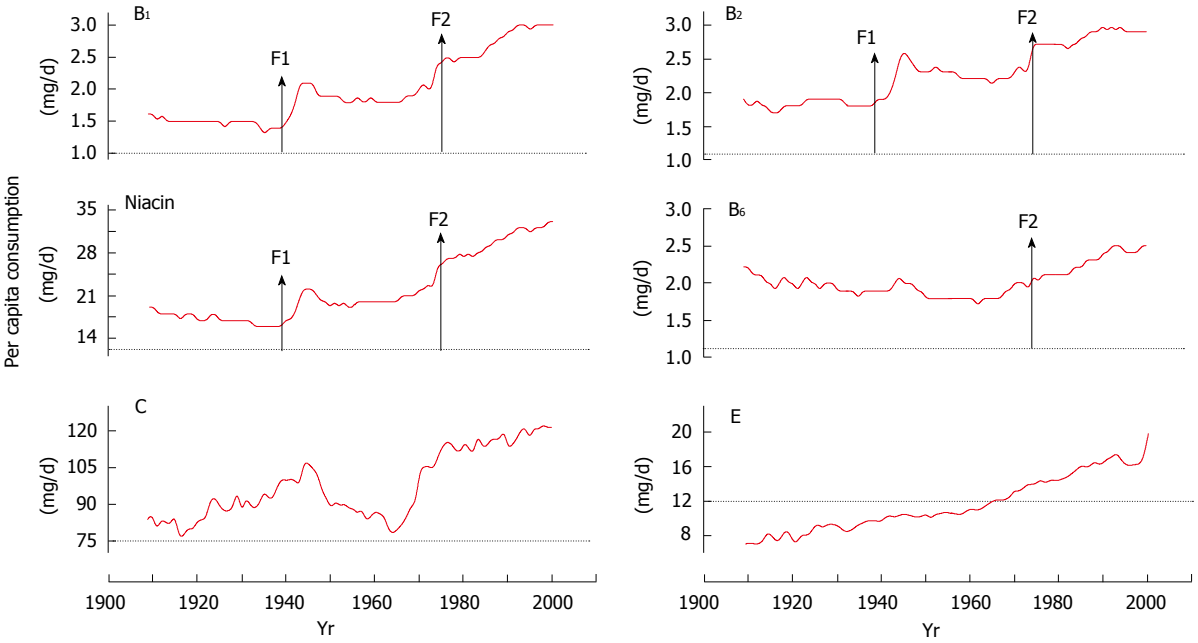

Figure 2 United States per capita daily vitamin consumption in 1909-2000. Data are from the Economic Research Service of the United States Department of Agriculture (http://search.ers.usda.gov/search?affiliate=ers&query=nutrients.xls). Red line indicates per capita consumption. Dot line indicates EAR. F1: Initiation of flour fortification; F2: Update of nutrient fortification standards for breakfast cereals in 1974[84]; EAR: The estimated daily average requirement.

Table 4 Obesity rate in selected countries with different wheat flour fortification policies

Country	Food policy	Standard (mg/kg flour, min)			Obesity rate in children
		Niacin	Vitamin B_1	Vitamin B_2	
Canada	Mandatory[1]	52.9	6.4	4	9-10[4]
United States	Mandatory[1]	52.9	6.4	4	6.8[5]
Kuwait	Mandatory[1]	52.9	6.4	4	14.6[6]
Saudi Arabia	Mandatory[1]	52.9	6.4	4	6-6.7[7]
United Kingdom	Mandatory[1]	16	2.4	0	5.1[5]
Finland	Prohibited[2]	0	0	0	2.5[5]
Norway	Prohibited[2]	0	0	0	2.2[5]
France	Prohibited[3]	0	0	0	1.6[5]

[1]Reference 36; [2]Reference 19; [3]Reference 42; [4]Reference 43, children (7-13 years) in 1996; [5]Reference 44, children (10 to 16 years) in 2001-2002; [6]Reference 45, children (10 to 14 years) in 2005-2006; [7]Reference 46 Children (1 to 18 years).

synthetic vitamins. The major differences include: (1) Different vitamin exposure among countries. Food fortification has caused significant differences in daily synthetic-vitamin consumption among countries due to different fortification policies and fortification standards[19], as shown in Table 4. Nationwide exposure to fortified foods in developing countries occurs much later than in developed countries[19], *e.g.*, it was not until 1994 that China began mandatory fortification[41]; (2) Different vitamin exposure among groups within countries. Wheat flour is fortified with B vitamins. Thus, those who use wheat flour products as staple foods possibly consume a higher amount of synthetic B vitamins. Vitamin-fortified foods are cheaper than fresh and natural foods in developed countries[34,47], which may lead to a higher intake of synthetic vitamins in low SES groups than in high SES groups in these countries[47,48]. In contrast, in developing countries, those who live in urban areas may consume more fortified foods than those who live in rural areas[49,50]. Infant formula milk (Table 3) and children foods (*e.g.*, ready-to-eat cereals[34]) are highly fortified with vitamins. Thus, infants fed formula milk and children are likely to have excess vitamin intake, as reported in the literature[51-54]; and (3) Different tolerance to fortified foods among population groups. Water-soluble vitamins can be eliminated through sweat[27,28]. Thus, under the same conditions of high vitamin intake, people who often sweat (*e.g.*, doing physical work and/or living in hot regions) may have a lower risk of excess accumulation of water-soluble vitamins in the body than those who rarely sweat (*e.g.*, living a sedentary life and/or in cold regions).

VITAMIN FORTIFICATION AND OBESITY PREVALENCE

Although there are few studies linking the increased prevalence of obesity to vitamin fortification, existing evidence suggests that high-risk populations are those who are most likely to have an increased intake of synthetic vitamins and decreased vitamin elimination, *e.g.*, populations in fortified countries[6], individuals with low SES in developed countries[6-10] or with high SES in developing countries[11,12,55], formula-fed infants[15-17], and those who live in fortified countries with less rigorous physical activity[56-59].

The prevalence of obesity varies from country to country. It seems that this variation may be related to dif-

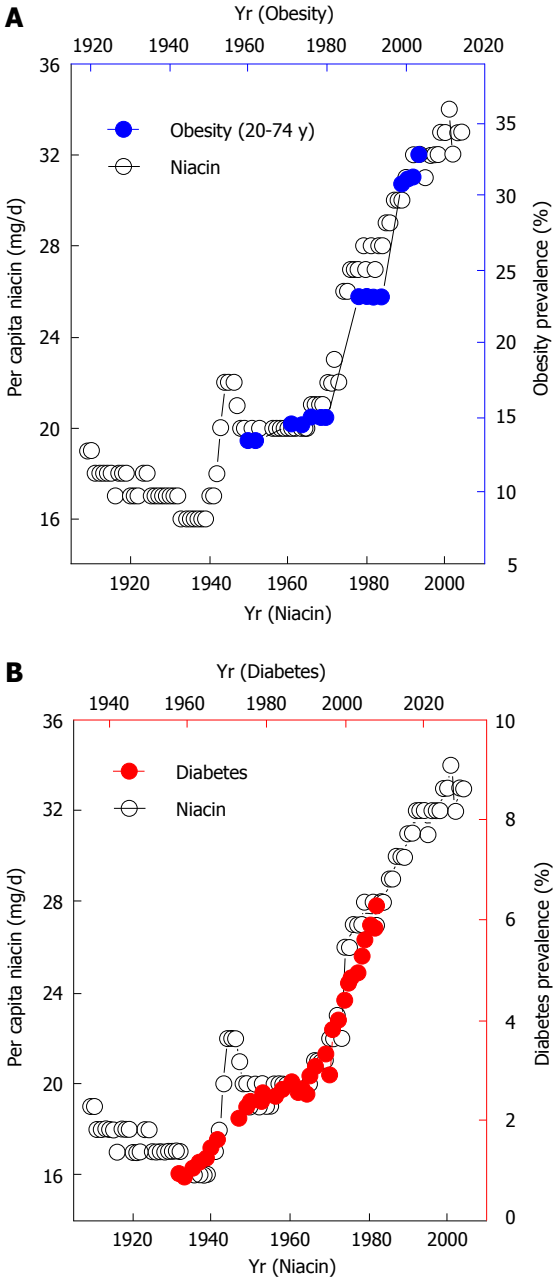

Figure 3 Lagged correlations between United States per capita niacin consumption and the prevalence of obesity and diabetes. The lag time between per capita niacin consumption and the prevalence of obesity and diabetes is 10 (A) and 26 years (B), respectively[25,26].

ferent food fortification policies and standards among countries. As shown in Table 4, the ranking of countries according to their prevalence of child obesity is similar to the ranking by the fortification standards of B vitamins. Evidently, flour fortification prohibited countries have a low prevalence of obesity, while countries with high flour fortification standard have high rates of obesity. Over the past few decades, food fortification has spread from developed countries to developing countries[19]. Therefore, it is possible that the spread of obesity from developed countries to developing countries may reflect the time sequence of implementing food fortification with vitamins.

Implementation of a vitamin fortification policy in a country will surely cause a sudden nationwide increase in vitamin intake in a short period. The initiation of food fortification with B vitamins in the late 1930s-1940s and the update of fortification standards in the 1970s in developed countries led to three phases in the consumption of vitamin B_1, B_2 and niacin: a rapid increase in the 1940s, followed by a plateau period between the 1950s and the 1960s and a steep increase thereafter, as shown in Figure 2. Available evidence has suggested an association between these food events and the prevalence of obesity. Two birth cohort studies conducted in Switzerland[60] and Denmark[61] showed that there was a significant increase in the prevalence of being overweight and obesity which occurred mainly in the cohorts born in the 1930s and the 1940s and in the cohorts born in the late 1960s to the 1970s. A Fels longitudinal study also showed that the child obesity epidemic in the United States is a sudden event that started in the 1970s and the 1980s[62]. A similar phenomenon is also seen in Saudi Arabia. Saudi Arabia started wheat flour fortification in the 1970s[63]. Following its food system change, Saudi Arabia experienced a rapid increase in obesity rates in the 1980s and the 1990s, and its obesity rate in schoolboys sharply increased from 3.4% in 1988 to 24.5% in 2005[64]. Our ecological studies clearly showed that there are strong lagged correlations between United States per capita consumption of B vitamins (B_1, B_2 and niacin) and the prevalence of obesity and diabetes[25,26]. Figure 3 clearly shows that both the initiation of food fortification in the 1940s and the update of fortification standards in 1974 are followed by a sharp increase in diabetes prevalence. The update of fortification standards followed a sharp rise in obesity prevalence.

As mentioned above, low SES groups in developed countries but high SES groups in developing counties may have a high synthetic vitamin intake from fortified foods. This may explain the findings that obesity is more prevalent in low SES groups in developed countries[6-10] but in high SES groups in developing countries[10-12,55]. Formula-fed infants have a high vitamin intake. Studies have demonstrated that formula-fed infants have a higher plasma level of vitamins compared with human milk-fed infants[51-53]. It is known that formula feeding[65-67] and micronutrient-fortified human milk feeding[68,69] can lead to rapid infant weight gain, a known major risk factor for children developing obesity[70-72]. Therefore, excess vitamin intake may mediate the link between formula feeding and childhood obesity.

In most developed countries, the energy expenditure needed for daily life has decreased since the beginning of the 20th century because of increasing mechanization, urbanization, motorization and computerization[4]. However, it is only since the 1970s, when food fortification standards were dramatically increased, that obesity prevalence has risen substantially. Moreover, although formula feeding is associated with an increased risk for obesity[15-17], there is no evidence indicating that there is a decrease in energy expenditure in formula-fed infants compared with breast-fed infants[73,74]. Instead, evidence shows that formula-fed infants may have higher total

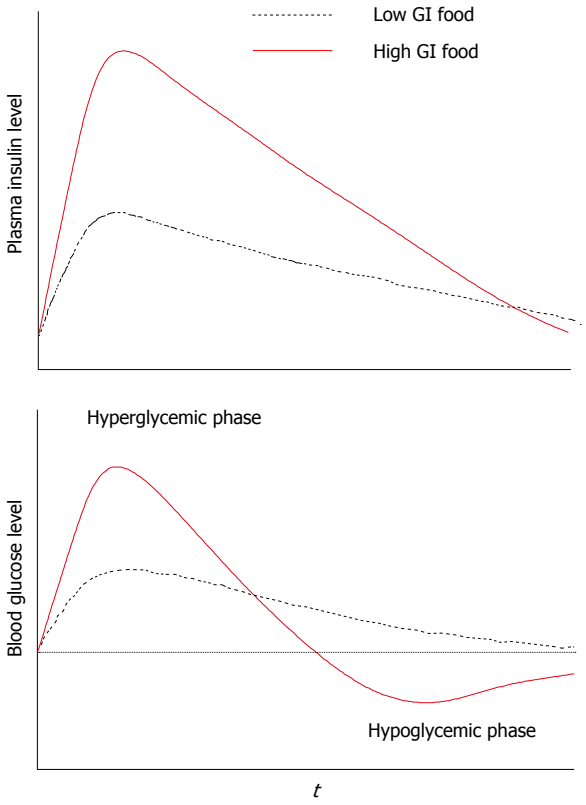

Figure 4 Typical glycemic responses to ingestion of a high glycemic index food and a low glycemic index I food. This figure is based on literature data[85-87]. GI: Glycemic index.

daily energy expenditure[13,14]. These data suggest that increased B vitamin intake rather than decreased energy expenditure may play a major role in the development of obesity. On the other hand, many studies, especially those conducted in highly B vitamin fortified countries, such as the United States[56], Canada[57], Saudi Arabia[58] and Kuwait[59], found that moderate to vigorous physical activity is associated with a reduced risk of obesity. It is proposed that this association may involve increased elimination of vitamins through sweat because moderate to vigorous physical activity can increase the sweat rate[28]. We have demonstrated that excess nicotinamide can be rapidly removed through sweating[75]. Sweat-mediated elimination of nicotinamide may be a crucial factor in preventing nicotinamide toxicity because human kidneys hardly excrete nicotinamide due to the reabsorption of renal tubules[76]. Therefore, it is conceivable that under the same conditions of high vitamin intake, those individuals who live a life that inhibits the activity of sweat glands (*e.g.*, physical inactivity) may be at greater risk of obesity. From this point of view, black people should be more sensitive to excess vitamins than whites, because the activity of sweat glands of blacks is lower than that of whites in the same temperature environment[77]. There is evidence showing that black women may have lower levels of physical activity than black men[78]. This may explain why obesity prevalence is greater in blacks, especially black women, than in whites in the United States[79,80]. Taken together, it may be concluded that food fortification-induced high intake of vitamins, especially B vitamins, may be responsible for the increased global prevalence of obesity.

MECHANISM OF EXCESS VITAMINS-INDUCED OBESITY

Many vitamins are known to act as coenzymes or as parts of enzymes responsible for essential chemical reactions, *e.g.*, the synthesis of fat and neurotransmitters. Excess vitamins may also affect the degradation of neurotransmitters and one-carbon metabolism. Therefore, excess vitamins may trigger obesity through multiple ways, including increasing fat synthesis, causing insulin resistance, disturbing neurotransmitter metabolism and inducing epigenetic changes.

B vitamins enhance fat synthesis

Obesity involves an accumulation of excess body fat. Early studies have already demonstrated that B vitamins play a crucial role in fat synthesis and there is a synergistic effect of B vitamins on fat synthesis. Vitamin B_1 and B_6 are required for the synthesis of fat from carbohydrate and protein[21-23] and their effects on fat synthesis are enhanced by the presence of other B vitamins. Vitamin B_6 administered together with B_1, B_2 and B_5 (pantothenic acid) resulted in a significant increase in body fat in rats[22]. Niacin has been found to increase daily feed intake, weight gain and percentage of abdominal fat in chicken when increasing supplementation from 0 to 60 mg nicotinic acid per kilogram diet[24]. It has been found that formula feeding leads to more fat gain, which may account for increased risk of later obesity[81,82]. Considering that formulas contain high levels of B vitamins (Table 3) that are a known factor increasing fat synthesis, we therefore propose that formula feeding-induced fat gain may be due to excess vitamins. Taken together, existing evidence suggests that excess vitamins, especially B vitamins, may play a role in the development of obesity.

Excess vitamins cause insulin resistance

Insulin resistance, a characteristic of obesity and type 2 diabetes[83], is a condition in which the tissues of the body do not respond appropriately to normal levels of insulin. It is known that glycemic and insulin responses are related to food. Foods can be classified by their glycemic index (GI, a relative measure of the incremental glucose response per gram of carbohydrate)[84]. Figure 4 shows the different glycemic and insulin responses to low GI food and high GI food. The typical glycemic response to high GI foods is a biphasic response, with an initial significantly higher blood glucose and insulin level (hyperglycemic phase) followed by significantly lower blood glucose level (postprandial reactive hypoglycemic phase)[85-87]. Postprandial reactive hypoglycemia stimulates appetite and may lead to increased caloric intake[86,88,89]. Therefore, it may be particularly important to understand how high GI foods induce a biphasic glycemic response.

Grain foods are a major source of carbohydrates.

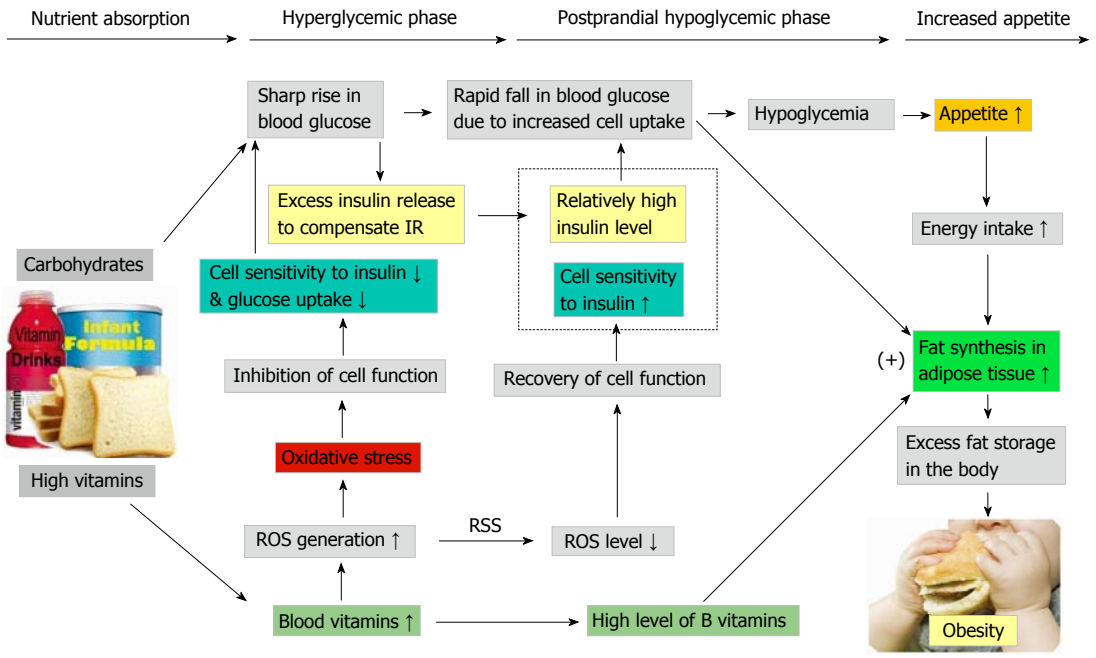

Figure 5 Proposed mechanism of excess vitamins-induced obesity. The absorption of sugar stimulates the release of insulin, while absorbed excess vitamins (from vitamin-fortified foods and drinks) generate ROS, leading to a decrease in the sensitivity of peripheral tissues to insulin (*i.e.,* insulin resistance). To compensate the insulin resistance, additional insulin has to be secreted, resulting in a high blood insulin level. Then, the sensitivity of peripheral tissues recovered with the rapid clearance of ROS, consequently, relatively high insulin level causes a rapid decrease in blood glucose due to increased glucose uptake, which may trigger excess energy intake. The conversion of glucose to fat in adipose tissue is promoted by high levels of B vitamins. Therefore, long-term consumption of vitamin fortified foods (including formulas) and drinks may cause fat accumulation in the body and subsequent obesity. ROS: Reactive oxygen species; RSS: ROS scavenging system.

Historically, high grain intake was associated with a low incidence of obesity. However, over the past few decades, refined (processed) grains became high GI foods[86,90,91]. Many processed grains (*e.g.,* white bread) produce even higher glycemic responses than simple sugars[86]. It seems that the effect of refined grains is not merely a matter of increased rate of digestion and absorption of carbohydrate, but a matter of increased insulin resistance. Grain foods are used as a vehicle of B vitamin fortification. Therefore, it is possible that the increased GI of processed grains may be due to their increased levels of B vitamins. Among the B vitamins fortified in foods, niacin is known to induce insulin resistance and glucose intolerance[92-95]. Nicotinamide is the most common form of niacin used in food fortification and infant formula supplementation (*e.g.,* Table 3). A study compared the glycemic and insulin responses of healthy subjects to glucose alone and glucose plus nicotinamide. The result showed that glucose plus nicotinamide significantly increased the levels of plasma insulin and hydrogen peroxide [a major component of reactive oxygen species (ROS)], followed by reactive hypoglycemia and hunger[26]. This study suggested for the first time that drinking nicotinamide-containing sugar-sweetened beverages may induce insulin resistance and nicotinamide fortification may contribute to the increased GI of refined grains.

It is known that increased ROS levels (*i.e.*, oxidative stress) may play a causal role in insulin resistance[96,97]. We therefore hypothesize that oxidative stress may mediate the effect of nicotinamide. The mechanism may be as follows. After glucose and nicotinamide are absorbed into the circulation, increased blood glucose level stimulates insulin secretion, while increased nicotinamide level may induce oxidative stress due to increased ROS generation (as found in Ref 26), leading to a decrease in cell functions, including insulin signaling (*i.e.*, insulin resistance). This results in a sharp increase in the level of blood glucose, which stimulates more insulin release (hyperglycemic phase). The clearance of ROS is more rapid than that of insulin. With the rapid clearance of ROS, cell response to insulin recovers quickly and as a result, the uptake of glucose by tissues (including adipose tissue) increases rapidly in response to relatively high insulin, which thus leads to a rapid fall in the level of blood glucose (hypoglycemic phase). Hypoglycemia initiates the feeling of hunger and subsequent feeding behavior. As mentioned above, B vitamins promote fat synthesis from carbohydrates. Thus, the cooperation of increased glucose uptake in the hypoglycemic phase and increased fat synthesis by high levels of B vitamins may induce excess fat storage and subsequent obesity (Figure 5). Unfortunately, the insulin resistance-inducing and obesity-promoting effects of B vitamins might have long been underestimated because traditional laboratory tests (*e.g.,* glucose tolerance test) are usually performed under fasting conditions, in which most, if not all, of increased ROS produced in the degradation of excess vitamins must have been cleared up after overnight fasting. For example, we found that oral nicotinamide (300 mg) induced increase in circulating hydrogen peroxide had returned to normal at 3 h[26].

It has been demonstrated in rats that the weight/fat gain-promoting effect of B vitamins is more efficient

when given in successive doses (added to the diet, like human food fortification) than in periodic doses[98]. This may explain why obesity prevalence significantly increased after the implementation of grain fortification with B vitamins. Because consumption of B vitamins-fortified foods may increase the burden of pancreatic islet B-cells, it is conceivable that obesity is closely associated with type 2 diabetes. In addition, other vitamins, even those that have antioxidant function (*e.g.,* vitamin C and E[99]), when used in large doses can increase ROS generation. Thus, high consumption of other vitamins may also contribute to the development of obesity. The relationship between dietary carbohydrates, excess vitamins, oxidative stress, insulin resistance, postprandial hypoglycemia, increased appetite and the development of obesity is proposed in Figure 5.

From the excess vitamin point of view, it may be easy to understand why the price of fast food, which determines the consumption of synthetic vitamins from fast food, may affect the body mass index of teens with low SES[100] and why vitamin-rich formulas[15-17] and sugar-sweetened beverages may increase the risk for obesity and type 2 diabetes[17,37,101,102]. It is interesting that some overweight children become overweight adults, while others do not[103]. One possible explanation for this may be a changing vitamin intake during the lifetime. Whether obese infants become obese children and then obese adults may to a large degree depends on the intake of vitamins after weaning. In theory, infants, even with normal body weight, may become obese adults if they always consume high vitamin-fortified foods (*e.g.,* refined grains) after weaning. We therefore recommend that the role of vitamin intake be taken into consideration in the study of the relationship between infant obesity and later obesity.

Excess vitamins may disturb neurotransmitter metabolism

Food intake is regulated by many neurotransmitters, including monoamine neurotransmitters (*e.g.,* dopamine and serotonin[104,105]) in the central nervous system. Therefore, factors that affect monoamine neurotransmitters may affect feeding behavior. Some vitamins are known to play an important role in the synthesis of monoamine neurotransmitters (serotonin and catecholamines). For example, vitamin B_6 is a cofactor for aromatic L-amino acid decarboxylase that catalyzes the formation of serotonin and dopamine[106]. Vitamin C enhances norepinephrine synthesis from dopamine by neuronal cells[107]. L-methylfolate, a derivate of the vitamin folate, also regulates the synthesis of the monoamine neurotransmitters serotonin, dopamine and norepinephrine[108].

Although small amount of vitamins can be directly eliminated through the urine, sweat[27,28,75] and sebum (such as vitamin E[29,30]), most of them usually undergo a series of phase I (oxidation, reduction and hydrolysis) and phase Ⅱ (conjugation, including glutathione conjugation, sulfation, methylation and glucuronidation) biotransformation before elimination from the body. As a result, vitamin degradation produces many metabolites. For example, at least 18 metabolites of vitamin B_1 are identified in the urine, of which six are major[109]. Niacin is degraded mainly to a number of methylated metabolites[110]. Vitamin C is degraded through sulfation[111] and glutathione conjugation[112]. Vitamin E also undergoes extensive metabolism and its conjugated metabolites (including sulfated) are also identified[113]. Because vitamins and neurotransmitters share the same biotransformation and detoxification system in the body[106,114], excess vitamins may affect the degradation of neurotransmitters by competing for the detoxification resources. For example, vitamin C has been known to inhibit the sulfation of other chemicals by competing for limited sulfate[111]. Although there are no systematic studies on the effect of vitamin fortification on the degradation of neurotransmitters, evidence has shown that excess vitamin C[115,116] and nicotinamide[117] can inhibit the degradation of catecholamines by depletion of sulfate and methyl groups, respectively. Thus, in theory, the effect of vitamins on the metabolism of monoamine neurotransmitters may affect the function of the nervous system. It is known that niacin can stimulate appetite. Niacin deficiency (*i.e.*, pellagra) is associated with a loss of appetite[118], which might involve changes in neurotransmitter metabolism in the brain.

Excess vitamins-induced obesity may involve epigenetic changes

Epigenetic changes are biochemical modifications that affect gene expression without changing the sequence of DNA. Emerging evidence suggests that epigenetic mechanisms may play a role in the development of obesity[119]. Epigenetic mechanisms involve an environment-gene interaction[120,121]. Nutrition is a crucial environmental factor which affects health and disease. Both maternal undernutrition and overnutrition can induce persistent changes in gene expression and metabolism[120]. Over the past few decades, one of the biggest changes in our food system has been the extensive use of synthetic vitamins. Therefore, it is possible that excess vitamin intake may contribute to epigenetic changes.

DNA methylation, which occurs at cytosine residues in CpG dinucleotides in gene promoters, is one of several epigenetic modifications[122]. The primary function of DNA methylation is to suppress gene expression. Global DNA hypomethylation increases genomic instability[122]. Although the mechanism of global DNA hypomethylation is not well understood, a lack of methyl groups may play a role in abnormal DNA methylation, because an adequate supply of methyl groups is a prerequisite for DNA methylation[123]. The biotransformation of some vitamins, especially niacin[117], may increase the demand for labile methyl groups and therefore, an excess intake of these vitamins may disturb DNA methylation by competing methyl groups. Recently, we tested this possibility by investigating the effect of nicotinamide supplementation on DNA methylation in rats and found that long-term high nicotinamide exposure led to a decrease in the methyl pool and in the levels of hepatic DNA methylation associated with alteration of gene expression[123]. Moreover,

maternal nicotinamide supplementation is also found to disturb fetal one-carbon metabolism in rats, including decreased global DNA methylation and decreased DNA uracil content in the brain and liver[124]. These data indicate that excess vitamins may be an important factor leading to epigenetic changes. The role of vitamin fortification in the development of methylation-related diseases is an open question.

NON-MONOTONIC EFFECT OF VITAMINS ON WEIGHT GAIN

Although it is known that B vitamins promote fat synthesis and vitamin-fortified foods and formulas increase the risk for obesity, why is there so little attention to the relationship between excess vitamin intake and obesity prevalence? A possible reason may be due to ignorance of the fact that the effect of vitamins on weight gain is non-monotonic. While vitamins are an important weight gain-promoting factor, at toxic levels they are no longer associated with weight gain or even cause weight loss.

It has long been known that many micronutrients (vitamins and minerals) are essential for life at low concentrations but become toxic at high concentrations. This phenomenon is termed Bertrand's rule[125]. The effect of vitamins on weight gain also follows this Bertrand's rule. We may take the weight-gain effect of niacin as an example. Jiang and colleagues[24] investigated the effects of dietary supplemental nicotinic acid at different doses (0, 30, 60 and 120 mg/kg diet) on the growth performance of chicken. They found that increasing supplementation from 0 to 60 mg nicotinic acid/kg tended to increase the average daily feed intake, weight gain and fat gain, *i.e.,* the maximum weight and fat gain was achieved at 60 mg/kg diet. Ivers and Veum found that among the doses used (6, 10, 14, 18, 22 and 44 mg/kg diet with adequate Trp), 14 mg of niacin/kg produced maximum weight gain in growing pigs[126]. Shibata *et al*[127] studied the effect of nicotinamide at doses of 0, 60, 1000 and 5000 mg/kg diet on rat weight gain. Their result showed that nicotinamide increased the food intake of rats, especially in the groups fed diet containing 60 and 1000 mg/kg of nicotinamide. The highest weight gain was observed at 60 mg/kg, while high-dose nicotinamide (5000 mg/kg diet) led to an inhibition of weight gain at the early stage of exposure due to its toxicity. These animal studies suggest that the supplemental dose for niacin to achieve maximum weight-gain effect may be around or less than 60 mg/kg diet. This dose is similar to that used in wheat flour fortification in some countries, *e.g.,* the United States, Canada, Saudi Arabia and Kuwait (Table 4). Thus, food fortification with niacin in these countries might have induced a maximum weight gain effect. In this case, further supplementation with niacin or niacin-containing multivitamin may offset the weight gain effect due to increased toxic effects, such as hepatotoxicity[128-131] and oxidative tissue damage[123]. This may account for the observations that further multivitamin supplementation in the United States[132] and Canada[133] or large-dose niacin treatment for dyslipidemia (1-3 g/d)[134,135] does not show weight gain.

Some other vitamins at high doses may also have toxic effects, including death. Davis *et al*[136] found that sudden infant death syndrome (a sudden and unexplained infant death) was association with high serum thiamin levels. A randomized controlled trial on vitamin C supplementation in very preterm infants showed that the infants who died in the trial were those who had significantly higher level of plasma vitamin C before randomization than surviving infants[137]. A systematic review and meta-analysis showed that long-term supplementation with beta carotene, vitamin A and vitamin E may increase mortality[138]. Therefore, it is not surprising that multivitamin supplementation in those who live in high-dose vitamin-fortified countries, *e.g.,* the United States[132] and Canada[133], may be associated with a slight weight loss. A similar phenomenon has been also observed in formula-fed infants. It has been found that formula feeding can lead to a more rapid weight gain, especially fat gain[81,82], compared to human milk feeding[17,65,67]. However, when formulas were further enriched with vitamins, their weight-gain effect was decreased rather than increased, compared with the standard formulas[139]. It seems clear that the weight-gain effect of vitamins has already been saturated at fortification doses used in infant formulas, children and adult foods, while further increasing the doses (*i.e.*, fortification plus additional supplementation) may induce a weight-loss effect due to the toxic effect. Considering that high vitamin intake which may cause hepatotoxicity (*e.g.,* niacin, as mentioned above) is very popular nowadays, we suggest that high vitamin intake may contribute to non-alcoholic fatty liver disease, the most frequent chronic liver disease in developed countries[140].

CONCLUSION

Since the late 1930s, when synthetic vitamins were first used, the human being has experienced the largest growth in vitamin intake in human history. It is possible that excess vitamins, especially B vitamins, may contribute to the development of obesity. Vitamin-rich formulas and food fortification with vitamins may, to a large extent, be responsible for the increased prevalence of obesity over the past several decades. Different fortification policies and standards may account for the differences in the prevalence between countries, while disparities in the consumption of fortified foods may contribute to the disparities in obesity between population groups within a country. Staple food fortification may be of great harm because it leads to a sustained high vitamin intake. Therefore, given that there has been a significant increase in vitamin supply from natural sources, it is necessary and urgent to review and modify the standards of vitamin fortification.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (1): Insulin

Insulin and bone: Recent developments

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Abstract

While insulin-like growth factor I is a well-known anabolic agent in bone evidence is beginning to accumulate that its homologue, insulin, also has some anabolic properties for bone. There is specific evidence that insulin may work to stimulate osteoblast differentiation, which in turn would enhance production of osteocalcin, the osteoblast-produced peptide that can stimulate pancreatic β cell proliferation and skeletal muscle insulin sensitivity. It is uncertain whether insulin stimulates bone directly or indirectly by increasing muscle work and therefore skeletal loading. We raise the question of the sequence of events that occurs with insulin resistance, such as type 2 diabetes. Evidence to date suggests that these patients have lower serum concentrations of osteocalcin, perhaps reduced skeletal loading, and reduced bone strength as evidenced by microindentation studies.

Key words: Type 2 diabetes; Insulin; Bone; Osteoblasts; Insulin resistance

Core tip: This is a review of recent publications that suggest an anabolic loop among bone, pancreas, and skeletal muscle.

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INTRODUCTION

The interactions between insulin and bone would on the surface appear to be an unlikely subject for an article, let alone a review article, but with the advent of the knockout mouse model many relationships that would not have been obvious now require investigation. The aim of this paper is to provide evidence supporting an anabolic loop including the pancreas, skeletal muscle, and bone.

GROWTH FACTOR

We do not want to confound the anabolic effects of insulin with those of insulin-like growth factor (IGF)-1, although the homology of molecular structure of both molecules may in fact account for some of the anabolic effects of insulin on bone. It should be emphasized at this point that insulin is synthesized in the pancreatic β cells while endocrine IGF-1 is synthesized in the liver. The stimuli for insulin production include glucose and, as we will see, osteocalcin, while endocrine IGF-1 is synthesized by liver in response to growth hormone and the paracrine IGF-1 produced by bone cells, including preosteoblasts and osteoblasts, osteocytes and osteoclasts[1,2] is synthesized in response to stimuli that have not yet been clarified.

While there are copious reports of the anabolic effects of IGF-1 on bone there is a growing amount of data suggesting that insulin itself has an anabolic effect on bone. Suggestions of this effect came from studies involving burned children in which a hyperinsulinemic, euglycemic clamp was employed resulting in an increase in both lean body mass, often indicative of muscle mass, and bone mass at time of hospital discharge compared

to controls, usually between 6 wk to 3 mo post-burn[3]. Moreover, both pre-osteoblasts and osteoblasts manifest different isoforms of the insulin receptor (IR), with IRA being expressed in pre-osteoblasts and IRB being expressed in mature osteoblasts[4]. This specificity suggests that insulin is a critical element in osteoblast differentiation from marrow stromal cells. This may have significance in the generation of the osteoblast peptide osteocalcin, which, as we shall see, has major implications for glucose metabolism. Whether the direct effect of insulin on osteoblasts has clinical significance, however, is not entirely clear. This is in part because the abovementioned report on hyperinsulinemia demonstrated increases in both lean body mass and bone mass[3].

INSULIN

The other side of this proposed loop is the effect of bone on insulin. The stimulus for the work that produced these findings is the knockout mouse model. In this model a significant contribution has been made by Wei *et al*[5] who have most recently reported that osteocalcin stimulates β cell replication in the pancreas *via* a cyclin D1-dependent mechanism utilizing the G-protein coupled receptor family C group 6 member A receptor expressed by these cells. This stimulation occurs during both peak β cell proliferation, which occurs in the perinatal period and in adult mice[5]. Moreover, they described the effects of daily osteocalcin injections in obese type 2 diabetic mice reporting an increase in the number of mitochondria in skeletal muscle as well as an increase in energy expenditure[6], indicating that osteocalcin can also increase muscle work by increasing insulin sensitivity.

Thus these recent data would suggest that under normal conditions insulin may stimulate osteoblast differentiation in order to produce more osteocalcin, which would then stimulate more insulin production by the pancreas and greater insulin sensitivity of skeletal muscle. There are also some recent clinical correlates of these studies in adults. In a recent study Díaz-López *et al*[7] performed a case-control study of 153 diabetic subjects and 306 individually matched controls and found that both the carboxylated and undercarboxylated forms of osteocalcin were lower than matched controls and that carboxylated osteocalcin concentrations were inversely associated with a model assessment of insulin resistance and fasting glucose concentrations. Another report by Gower *et al*[8] indicated that in obese individuals total osteocalcin was directly associated with skeletal muscle but not hepatic insulin sensitivity while undercarboxylated osteocalcin was associated with β cell function in those with abnormal fasting glucose concentrations.

BONE

A major unanswered question is exactly what happens to bone in cases of peripheral insulin resistance? Are the IRs in pre-osteoblasts and osteoblasts down-regulated? We know that osteocalcin levels are lower in type 2 diabetics[7,8]. In addition, we know that insulin resistance is also caused by factors that cause bone resorption, such as the interleukin-6-mediated chronic low grade inflammation that contributes to non-alcoholic fatty liver disease (NAFLD)[9] and excessive glucocorticoid production, another significant contributor to NAFLD[10]. However, we do not at this point know precisely how peripheral insulin resistance affects bone. One conjecture would be that if muscles expend less energy due to their inability to take up glucose then muscle strength may be reduced and skeletal loading may also be consequently decreased. This scenario could explain abnormalities in bone with type 2 diabetes. Were this to be so then bone loss would result in reduced production of osteocalcin and a perpetuation of the problem of peripheral insulin resistance.

So, why has bone loss with type 2 diabetes been so difficult to determine up to now? As summarized by Ferrari[11] in a review article on diabetes and osteoporosis, bone mineral density (BMD) may not be reduced in this condition inasmuch as weight and fat mass must be factored into the BMD determinations. The probability of fracture as assessed by use of the on-line FRAX tool developed by the World Health Organization may also underestimate fracture risk in this condition. As evidence that this may indeed be the case a recent report by Hothersall *et al*[12] examined the files of all hip fractures in Scotland from 2005-2007 and the prevalence of both type 1 and type 2 diabetes in this population. While there was a significant correlation between hip fractures and type 1 diabetes, in which insulin deficiency is the issue, there was no overall increased risk of hip fracture in type 2 diabetes, according to this review. The investigators do, however, state that these findings do not rule out increased risk in sub-groups of type 2 diabetics. While we have demonstrated that osteocalcin, also a marker of bone formation, is lower in patients with type 2 diabetes, not all markers of bone formation or resorption are consistent. For example, Chen *et al*[13] found that while osteocalcin was lower in diabetics *vs* controls, there was no difference in bone specific alkaline phosphatase. Similarly while Bhattoa *et al*[14] found that urinary cross-laps, a resorption marker, was lower in type 2 diabetics *vs* controls while Chen *et al*[13] found that urinary hydroxyproline was elevated.

A new development, however, has shed some light on this problem. In a study that has been Epublished ahead of print, Farr *et al*[15] have reported the use of *in vivo* microindentation of the tibia as an index of bone strength. In this study of 60 post-menopausal women, half of whom had type 2 diabetes, this technique demonstrated decreased bone strength in the diabetic women.

Much more work needs to be done to follow up on these findings but clearly the greater chance of microcracks in the bones of insulin-resistant diabetics may not be detected by bone density determinations.

Therefore, for those who care for diabetic patients, the complications involving bone have been subtle and difficult to detect but as more attention is being paid to this area the pathogenesis of the bone problem should

be more clearly elucidated and new therapeutic targets identified.

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REVIEW

Cardiac autonomic neuropathy in patients with diabetes mellitus

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Abstract

Cardiac autonomic neuropathy (CAN) is an often overlooked and common complication of diabetes mellitus. CAN is associated with increased cardiovascular morbidity and mortality. The pathogenesis of CAN is complex and involves a cascade of pathways activated by hyperglycaemia resulting in neuronal ischaemia and cellular death. In addition, autoimmune and genetic factors are involved in the development of CAN. CAN might be subclinical for several years until the patient develops resting tachycardia, exercise intolerance, postural hypotension, cardiac dysfunction and diabetic cardiomyopathy. During its sub-clinical phase, heart rate variability that is influenced by the balance between parasympathetic and sympathetic tones can help in detecting CAN before the disease is symptomatic. Newer imaging techniques (such as scintigraphy) have allowed earlier detection of CAN in the pre-clinical phase and allowed better assessment of the sympathetic nervous system. One of the main difficulties in CAN research is the lack of a universally accepted definition of CAN; however, the Toronto Consensus Panel on Diabetic Neuropathy has recently issued guidance for the diagnosis and staging of CAN, and also proposed screening for CAN in patients with diabetes mellitus. A major challenge, however, is the lack of specific treatment to slow the progression or prevent the development of CAN. Lifestyle changes, improved metabolic control might prevent or slow the progression of CAN. Reversal will require combination of these treatments with new targeted therapeutic approaches. The aim of this article is to review the latest evidence regarding the epidemiology, pathogenesis, manifestations, diagnosis and treatment for CAN.

Key words: Diabetes mellitus; Cardiac; Cardiovascular; Autonomic; Neuropathy; Dysfunction; Cardiac autonomic neuropathy; Sympathetic; Parasympathetic; Heart rate variability; Spectral analysis; Diabetic cardiomyopathy; Postural hypotension

Core tip: Cardiac autonomic neuropathy (CAN) is a complication of diabetes mellitus that is often underdiagnosed but can lead to severe morbidity and mortality, due to the associated cardiovascular burden. New evidence has emerged surrounding its complex pathways, but its full pathogenesis is yet to be understood. CAN manifests in a spectrum of subclinical and clinical presentations, ranging from resting tachycardia to cardiomyopathy. Heart rate variability and scintigraphy have enabled the diagnosis at a subclinical stage, thus providing the opportunity for better prevention and treatment. However, no definite therapeutic approaches have been adopted to date, emphasizing the need for newer targeted treatments.

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INTRODUCTION

Diabetes mellitus (DM) is a global epidemic affecting at least 8.3% of the global population and 371 million people worldwide with a significant proportion (50%) remaining undiagnosed. It is estimated that almost one in six people are currently at risk of developing diabetes-related complications[1]. Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with diabetes and subsequently the primary goal of diabetes treatment is to reduce the burden of CVD as well as the vascular complications associated with diabetes[2,3]. Much of the CVD prevention strategies in patients with DM are based on lowering blood pressure and LDL-cholesterol levels and improving glycaemic control[4-7]. Despite that, CVD remains very common and a major cause of mortality and morbidity in patients with DM. Hence, better understanding of pathogenesis of CVD is crucial to develop new therapeutic targets.

Cardiac autonomic neuropathy (CAN) is a very common and often overlooked diabetes-related complication that has a major impact on CVD, mortality and morbidity in patients with DM[8,9]. Improving our understanding of the pathogenesis of CAN and its role in CVD, offers the potential of new treatment targets that might reduce the burden of CVD in patients with diabetes. This review aims to provide an overview of the epidemiology, pathogenesis, cardiovascular consequence, diagnosis, and treatments of CAN, with particular emphasis on the latest developments in the field.

LITERATURE SEARCH STRATEGY

We conducted a review of the original papers and review articles indexed in PubMed, Medline and Google Scholar between 1975 and 2013. We have used several terms individually or in combination including: diabetes, autonomic neuropathy, CAN, cardiovascular, cardiac, autonomic, neuropathy, dysfunction. Only articles in English and in adult population were reviewed.

DEFINITIONS AND EPIDEMIOLOGY

Based on the CAN Subcommittee of the Toronto Consensus Panel on Diabetic Neuropathy[10], CAN is defined as the impairment of cardiovascular autonomic control in patients with established DM following the exclusion of other causes. CAN, especially at the early stages, can be sub-clinical and thus as the disease progresses, it becomes clinically evident.

The prevalence of CAN varies between 1%-90% in patients with type 1 DM (T1DM) and 20%-73% in patients with T2DM (Table 1). This huge variation in CAN prevalence is due to the inconsistency in the criteria used to diagnose CAN and significant differences in the study populations, particularly in relation to CAN risk factors (such as age, gender and DM duration amongst others).

CAN has been detected at time of diagnosis of diabetes in patients with either T1DM or T2DM irrespective of age, suggesting that CAN presentation is not limited by age or type of diabetes and can occur before DM is evident clinically[11-15]. However, the duration of diabetes is an independent factor for developing CAN irrespective to diabetes type[10,16]. CAN is detected in about 7% of both T1DM and T2DM at the time of initial diagnosis[17], and it is estimated that the risk for developing CAN increases annually by approximately 6% and 2% in patients with T1DM and T2DM respectively[17-19].

Poor glycaemic control is a major risk factor for CAN progression[14,19-21]. In the Diabetes Control and Complications Trial (DCCT), intensive glycaemic control resulted in a 50% decrease in CAN incidence over the 6.5 years follow-up period[19]. This protective effect persisted 14 years after the end of the study despite the disappearance of HbA1c differences that were achieved between the groups during the randomised phase of trial[18]. Similarly, CAN has been shown to be associated with conventional CVD risk factors, such as hypertension, smoking, hyperlipidaemia and obesity[22-24]. In the Steno-2 trial of patients with T2DM and microalbuminuria, intensive pharmacological intervention targeting hypertension, hyperlipidaemia and microalbuminuria combined with behavioural treatment (exercise, diet and smoking cessation) reduced the risk of autonomic neuropathy over the course of a 7.8 years follow-up (HR = 0.37, 95%CI: 0.18-0.79)[5]. After a mean of 5.5 years following the end of the study, the same protective effect against the development of autonomic neuropathy persisted (RR = 0.53, 95%CI: 0.34-0.81, P = 0.004). There was also reduction in the risk for developing CVD (RR = 0.43, 95%CI: 0.19-0.94, P = 0.04) and overall mortality (RR = 0.54, 95%CI: 0.32-0.89, P = 0.02) in this study[25].

Moreover, in a large cohort of more than 1000 patients with T2DM the incidence of CAN over a 7.5 years follow-up correlated with age (P < 0.001) and microvascular disease (P = 0.035)[26]. Diabetic nephropathy (including microalbuminura), diabetic retinopathy and diabetic polyneuropathy have been widely identified as clinical predictors of CAN[23,24,27], which is not surprising as diabetic microvascular complications share common mechanisms and risk factors. The impact of gender on CAN is controversial. In a multi-centre, cross sectional study of 3250 patients with DM, CAN prevalence was no different between men and women (35% male *vs* 37% female)[28]. However, in the action to control cardiovascular risk in diabetes trial including more than 8000 patients with T2DM CAN was more prevalent in women (2.6% in men *vs* 4.7% in women for moderate severity CAN and 1.4% in men *vs* 2.2% in women for severe CAN, P < 0.01 for all three definitions of CAN in the study)[29].

Table 1 Prevalence of cardiac autonomic neuropathy as reported in major studies

Ref.	Year	Country	*N* of subjects	Type of DM	Population characteristics	Diagnostic test	Criteria applied	Prevalence (%)	Comments
O'Brien *et al*[111]	1991	United Kingdom	506	IDDM	Mean age 45 yr, mean DM duration 15 yr, female 42%	HRV in response to (1) rest (2) single deep breath (3) Valsalva manoeuvre or (4) standing	At least two positive of the tests mentioned in the previous column	17	Prevalence of CAN was associated with the presence of other DM complications
Ziegler *et al*[223]	1992	Germany Austria Switzerland	130 647 524	Newly diagnosed IDDM Total IDDM Non-IDDM		CV of HRV, low- and mid-frequency bands of spectral analysis, MCR, Valsalva manoeuvre or lying-to standing	At least three positive of the tests mentioned in the previous column	7.7 25.3 34.3	
Kennedy *et al*[11]	1995	United States	290	IDDM	Listed pancreas transplantation recipients	HRV Valsalva manoeuvre		90 88	
DCCT research group[19]	1998	United States	1441	IDDM (1) primary prevention cohort (absence of end–organ damage such as retinopathy and microalbuminuria) (2) secondary intervention cohort (mild/moderate retinopathy +/- microalbuminuria)	Mean age 27 yr, female 47% duration of DM 1-5 yr (mean 2.6) primary prevention cohort 1-15 yr (mean 8.8) secondary intervention cohort	HRV Valsalva manoeuvre Postural BP	R-R variation < 15 Valsalva ratio < 1.5 Diastolic BP drop > 10 mmHg	1.6-6.2 5.5-6.3 0	These figures represent baseline characteristics
Kempler *et al*[28] (EURODIAB IDDM)	2002	16 European countries	3250	T1DM	Mean age 32 yr, mean DM duration 14 yr, female 49%	(1) R-R response to standing (2) Postural BP	R-R ratio < 1.04 or drop > 20 mmHg in systolic BP	36	Correlation with age, DM duration and HbA1c
Gaede *et al*[5,224] (the Steno type 2 study)	2003	Denmark	160	T2DM	Mean age 55 yr, female 27%, HbA1C 8.8% at baseline	(1) R-R response to breathing (2)Postural BP	R-R variation < 6 or drop > 25 mmHg in systolic BP	27.5	This figure represents baseline findings
Valensi *et al*[27]	2003	France	245 151	T1DM T2DM	Mean age 39.6 yr, mean DM duration 8.6 yr, female 43%	R-R response to (1) deep breathing (2) Valsalva and (3) standing	Criteria for abnormal tests were based on Armstrong *et al*[225] At least two positive tests (classed as moderate CAN)	21.2 20.7 33.5 20	Rate of moderate/severe CAN was higher in T1DM (18.2% and 4.8%) than in T2DM (12.3% and 2.3%) (P = 0.031)
Low *et al*[23]	2004	United States	83 148	T1DM T2DM	Mean age 59 yr, white 99%, female 48%	(1) Sudomotor axon-reflex test (2) Valsalva manoeuvre (3) BP and HR response to standing (4) R-R response to deep breathing	CASS ⩾ 1 in two domains or ⩾ 2 in one domain (sudomotor, cardiovagal, adrenergic)	54 73	This study focuses on DAN but encompasses several cardiac autonomic tests
Pop-Busui *et al*[18] (DCCT/EDIC study)	2009	United States	620 591	IDDM-former intensive Tx group IDDM-former conventional Tx group	Mean age 47 yr in both groups, mean DM duration 26 yr, female 49% and 46% respectively	R-R response to (1) deep breathing (2) Valsalva manoeuvre (3) postural BP	R-R < 15 or R-R 15-19.9 and Valsalva ratio < 1.5 or drop > 15 mmHg in diastolic BP	29 35	13/14 yr post closeout of DCCT

DM: Diabetes mellitus; IDDM: Insulin dependent diabetes mellitus; CV: Coefficient of variation; MCR: Mean circular resultant; HRV: Heart rate variability; BP: Blood pressure; CAN: Cardiac autonomic neuropathy; CASS: Composite Autonomic Severity Score; DCCT: Diabetes Control and Complications Trial; T1DM: Type 1 diabetes mellitus.

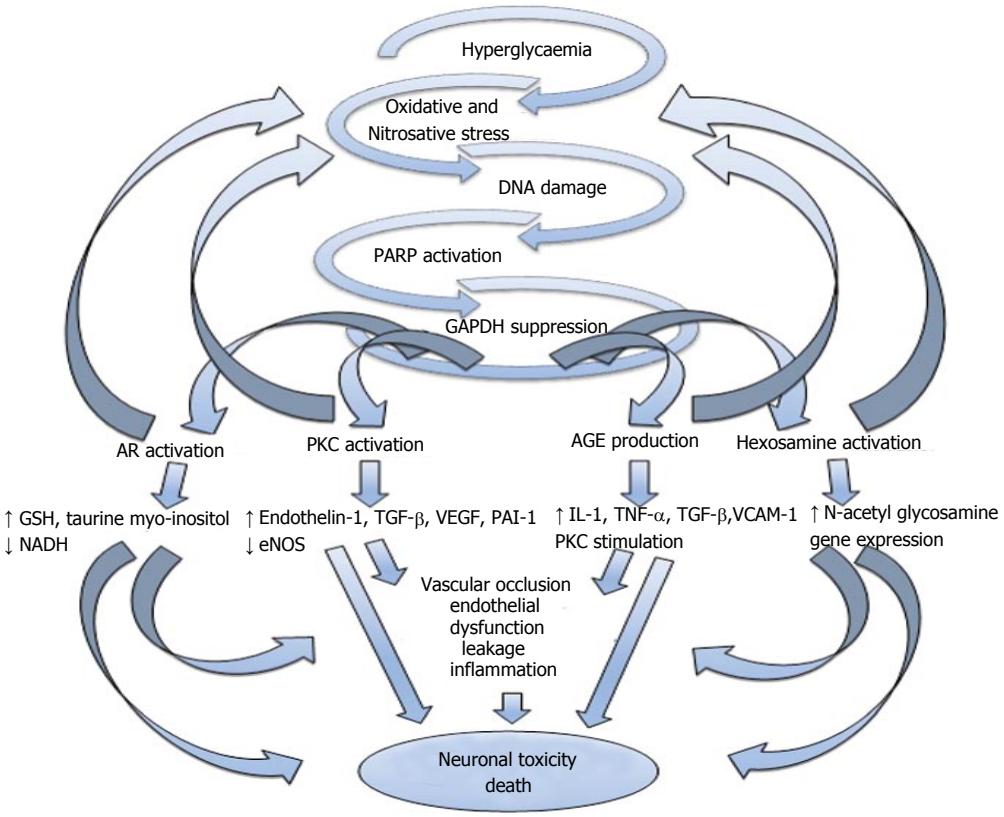

Figure 1 Summary of the mechanisms that relate hyperglycaemia to microvascular complications in patients with diabetes. PKC: Protein kinase C; AGE: Advanced glycation end-products; PARP: Poly ADP-ribose polymerase; GAPDH: Glyceraldehyde-3 phosphate dehydrogenase; GSH: Glutathione; NADH: Nicotinamide adenine dinucleotide; TGF-β: Transforming growth factor; VEGF: Vascular endothelial growth factor; PAI-1: Plasminogen activator inhibitor-1; eNOS: Endothelial nitric oxide synthase; IL-1: Interleukin 1; TNF-α: Tumour necrosis factor-α; VCAM-1: Vascular cell adhesion molecule 1.

Ethnicity has also been postulated to be a risk factor for CAN as South Asians seem to have lower rates of peripheral neuropathy than White Europeans with DM[30]. More specifically, the prevalence of small fibre neuropathy was significantly lower in Indian Asians than in Europeans (32% *vs* 43% respectively, $P = 0.03$) and mean nerve conduction velocity Z scores (measuring large fibre neuropathy) were superior in Asians compared to Europeans (mean ± SD 0.07 ± -0.62 *vs* -0.11 ± 0.60, $P = 0.007$). However, using heart rate variability (HRV) spectral analysis as well as frequency and time domain analysis showed no difference in CAN prevalence between South Asians and white Europeans (Tahrani *et al*, unpublished data).

PATHOGENESIS OF CAN

The exact pathogenesis of CAN is complex and remains unclear. Most of the proposed mechanisms of neuronal injury are based on models of somatic rather than autonomic neuropathy. Although many of these mechanisms might be shared between autonomic and somatic neuropathies, differences do exist as shown by the Steno-2 trial (described above) in which the multi-factorial intervention (including intensive metabolic control and lifestyle changes) slowed down the progression of autonomic but not somatic neuropathy.

Hyperglycaemia induced neuronal injury and ischaemia

The pathogenesis of CAN is likely to be multi-factorial[31] and to involve several mechanisms and pathways that lead to neuronal ischaemia or direct neuronal death/dysfunction (Figure 1). Hyperglycaemia and the adverse metabolic environment in patients with DM result in increased oxidative and nitrosative stress[17], which can cause direct neuronal damage/dysfunction as well as endothelial dysfunction resulting in neuronal ischaemia. Neuronal axons are rich in mitochondria which makes them particularly susceptible to the direct and indirect effects on oxidative and nitrosative stress[32].

Increased oxidative stress results in poly ADP-ribose polymerase activation which when coupled with other activated downstream pathways including the polyol pathway, advanced glycation endproducts production, protein kinase C and the hexosamine pathway are thought to contribute to glucose toxicity[33-36]. These different pathways can in return exacerbate oxidative stress and can induce changes in gene expression, transcription factors, diverse cellular products disrupting several cellular functions and the communication between the cell and the surrounding matrix all of which leads to neuronal dysfunction and death[37-39]. These pathways also result in impaired microvascular-- regulation and endothelial dysfunction by different mechanisms, including increase in plasminogen activator inhibitor-1 and endothelin-1 production and impairment of endothelial nitric oxide (NO) synthase and NO actions[40,41]. This can lead to reduction of neurovascular perfusion, dysfunction and cellular apoptosis[42].

Autoimmunity

The role of autoimmunity has also been explored particularly in patients with T1DM. The presence of complement-fixing antibodies against sympathetic and para-

sympathetic tissues in patients with insulin-dependent diabetes and their correlation with CAN was described in the early 90s[43,44]. In a study of 78 patients with DM, the prevalence of phospholipid autoantibodies (PLA) in the patient's serum was significantly higher in those tested positive for autonomic neuropathy (88% of the patients with autonomic neuropathy *vs* 32% of those without, $P < 0.001$) and there was a strong correlation between the PLA titre and total neuropathy score ($r^2 = 0.58$, $P = 0.0002$)[45]. Granberg *et al*[46] demonstrated in a group of patients with T1DM that patients positive for complement-fixing antibodies to the sympathetic ganglion, vagus nerve and adrenal medulla had a significant higher risk to develop cardiac autonomic dysfunction (measured by the E/I ratio during deep inspiration and HRV to postural change) over a 6-year follow-up (RR = 7.5, 95%CI: 1.72-32.80). There are, however, conflicting reports whether these auto-antibodies contribute to the pathogenesis of autonomic neuropathy or represent rather incidental findings and can be attributed to autoimmunity against concurrent conditions, such as thyroid disease[47]. A recent study of mixed T1DM and T2DM patients concluded that neither peripheral nor CAN was associated with the presence or the levels of Neuropeptide Y Autoantibodies[48].

Residual β-cell function

Several studies have shown a protective effect of residual β-cell function (*i.e.,* C-peptide levels) on the development and incidence of microvascular complications (including CAN) in patients with T1DM[49,50]. The exact mechanisms for these associations are not clear but it is thought that the C-peptide activates Na/K channels, lowers inflammation and improves NO bioavailability and endothelial function[51,52]. Small RCTs have shown beneficial effect of C-peptide treatment on CAN parameters[53].

Genetic factors

More recently data suggesting genetic predisposition to CAN have emerged. In a study of 154 patients with T2DM, TCF7L2 gene was found to be strongly associated with the presence of CAN, as assessed by deep breathing, lying to standing, Valsalva manoeuvre and postural hypotension tests (OR = 8.28, $P = 0.022$ for the rs7903146 allele)[54]. Another study on healthy Japanese individuals showed that the T393C polymorphism of the gene encoding the Gs-protein-α-subunit (GNAS1) is significantly associated with cardiovascular autonomic dysfunction, detected with power spectral analysis ($P < 0.05$ for TT + TC *vs* CC polymorphism)[55]. Twins studies, however, failed to show an association between CAN and genetic factors[56].

Obstructive sleep apnoea

Obstructive sleep apnoea (OSA) is emerging as another possible factor in the development of CAN. OSA is very common in patients with diabetes and has been associated with increased sympathetic tone in patients without diabetes[57,58]. The interrelationship between OSA and CAN in patients with DM requires further investigation and is likely to be bidirectional. While the intermittent hypoxia that occurs in OSA could lead to increased oxidative stress, nitrosative stress, and impaired microvascular complications which could lead to CAN[59], CAN on the other hand could lead to changes in upper airways tone and changes in respiratory drive which could predispose the patient to OSA. One recent study presented in the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes 2012 meeting showed that the prevalence of CAN was similar in patients with T2DM with and without OSA, but CAN severity was worse in the OSA group (Tahrani *et al*[59], unpublished data). Furthermore, the presence of CAN was associated with more severe apnoea/hypopnea episodes (Tahrani *et al*[59], unpublished data).

NATURAL HISTORY OF CAN

DM affects the autonomic (as well as the peripheral) nervous system in an ascending length-dependent manner. The vagus nerve, which anatomically is the longest autonomic nerve and physiologically mediates 75% of the overall parasympathetic activity, tends to be involved early in the course of CAN development. The early stages of CAN therefore involve reduction in parasympathetic activity, which results in sympathetic predominance. This increase in sympathetic tone continues until the latest stage of CAN when sympathetic denervation ensues, which spreads gradually from the apex to the base of the heart[60,61].

CAN is divided into a sub-clinical and a clinical stage. During the initial sub-clinical stage, CAN is detected through abnormalities in frequency and time domains of the spectral analysis of HRV and the Baroreflex Sensitivity (BRS) tests, as well as an increased torsion of the left ventricle (LV) on cardiac imaging before the development of abnormalities in standard cardiac autonomic reflex testing (CART) (please see below for details)[62-67]. Studies have shown that these abnormalities can even be present at the time of diagnosis of DM[63]. CAN progresses and parasympathetic denervation is followed by compensatory sympathetic overdrive, resulting in abnormal CARTs followed by symptomatic CAN in which the clinical manifestations become apparent (please see below). At the stage of sympathetic denervation, autonomic dysfunction correlates clinically with postural hypotension[63] (Figure 2). The time scale for the progression of subclinical CAN to the development of abnormal CART is unclear; similarly the natural history of the development of early cardiac abnormalities (such as torsion or deficits in myocardial perfusion or cardiac energetic) and its relationship to subclinical CAN is also unclear. But we estimate that many patients with sub-clinical CAN will develop abnormal CART and early features of cardiac involvement within 5 years of developing abnormal frequency and time domain parameters.

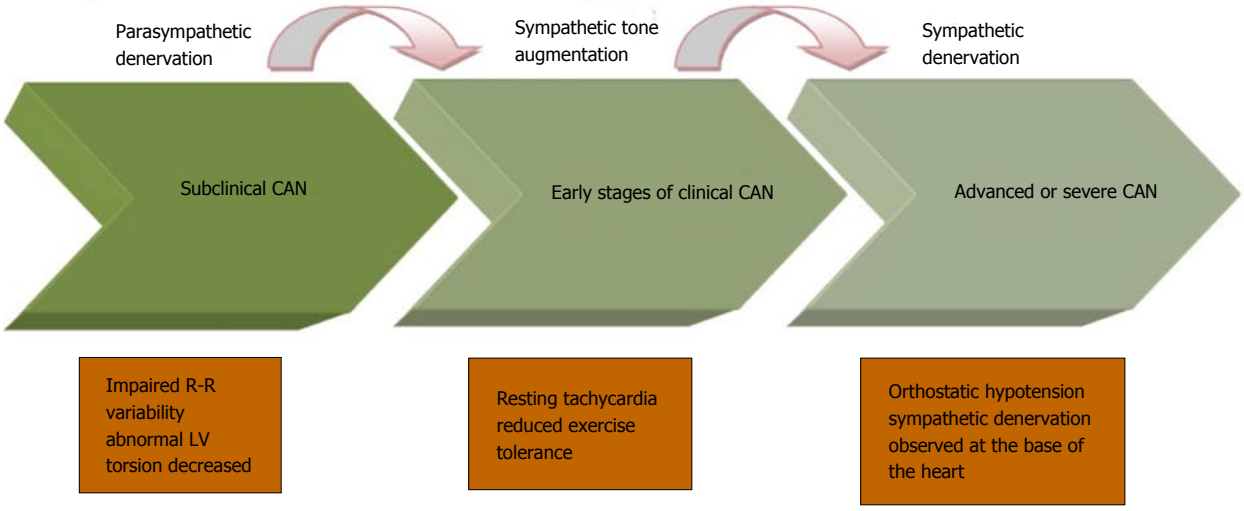

Figure 2 Natural progression of CAN and correlation with clinical signs and symptoms. CAN: Cardiac autonomic neuropathy; LV: Left ventricle.

CLINICAL MANIFESTATIONS OF CAN

Resting tachycardia

Resting tachycardia is a common manifestation of CAN that occurs at a relatively early stage of the disease. A HR of 90-130 beats per minute (bpm) can be observed and is associated with a reduction in parasympathetic tone followed by increased sympathetic activity as CAN progresses[68]. A fixed HR which does not change during sleep, exercise or stress is a sign of complete cardiac denervation[69]. Moreover, poor HR response to adenosine is associated with higher risk for adverse cardiac events[70], including all-cause and CVD mortality[71]. Hence, resting HR can be used as a diagnostic and prognostic tool in patients with DM after excluding other causes of tachycardia[10].

Exercise intolerance

Impaired blood pressure, HR and cardiac stroke volume in response to exercise in the absence of structural or coronary cardiac disease are all features of CAN[69]. As disease progresses, the parasympathetic-sympathetic imbalance can lead to further impairment of the above parameters[68] which limits the diagnostic utility of exercise tolerance testing in these patients due to increased false-negative outcomes caused by blunted HR response[72]. In addition, patients with CAN should be tested using stress cardiac imaging (usually echocardiography) prior to starting an exercise program, especially those with high-risk profile[69].

Orthostatic hypotension

Orthostatic hypotension is a manifestation of advanced CAN. Orthostatic hypotension is defined as the reduction in systolic blood pressure by > 20 mmHg or in diastolic blood pressure by > 10 mmHg 2 min following postural change from supine to standing[17,19,69]. Orthostatic hypotension occurs as a result of the impairment of the sympathetic response to postural change secondary to poor norepinephrine response and abnormalities in the baro-receptor sensitivity, resulting in inadequate HR response and peripheral vasoconstriction[23,69]. Orthostatic hypotension can be aggravated by many medications that are commonly used in patients with DM such as diuretics, vasodilators, tricyclic antidepressants and insulin[63]. Similar to resting tachycardia, assessing the presence of orthostatic hypotension is of prognostic value as a marker of advanced CAN[10]. In the middle-aged general population, orthostatic hypotension has been shown to be an independent prognostic factor for CVD and all-cause mortality[73].

Silent ischaemia

CAN is associated with a prolonged subjective angina threshold (which is defined as the time between the observation of 1 mm ST depression on the electrocardiogram and the development of symptoms of angina pectoris); thus rendering patients with CAN susceptible for experiencing silent myocardial ischaemia and potentially infarction, despite being asymptomatic[74]. A meta-analysis of 12 cross-sectional studies showed that CAN is associated with silent ischaemia in patients with DM (the Mantel-Haenszel estimate for prevalence rate risk was 1.96, 95%CI: 1.53-2.51)[17]. A study of 120 patients with DM and no previous CVD found evidence that CAN (detected using the Valsalva manoeuvre, the deep breath test and lying-to-standing HRV) was a better predictor of major cardiac events [*i.e.,* myocardial infarction or myocardial infarction (MI)] than the presence of silent ischaemia (OR = 4.16, 95%CI: 1.01-17.19) but when CAN was combined with silent ischaemia the risk was even higher (5 out of 10 had a major event)[75]. A study from Spain that included 217 patients with T1DM and T2DM, found that the presence of autonomic neuropathy is independently associated with increased risk for developing silent ischaemia (as demonstrated by positive exercise test) (OR = 6.5, 95%CI: 1.3-7.9) especially when combined with other cardiovascular risk factors such as microalbuminuria[76]. In the Detection of Ischaemia in Asymptomatic Diabetic subjects study which included 1123 patients with T2DM,

CAN (defined as abnormal Valsalva manoeuvre) was also a predictor of silent ischaemia (defined using stress cardiac perfusion imaging) (OR = 5.6, 95%CI: 2.6-12.4, P = 0.0001)[77].

It is evident that patients with DM and CAN are at high risk of sustaining a major cardiovascular event during exercise, due to the limited perception of ischaemic pain which could delay the appropriate and timely response to ischaemia. A recent statement from the Toronto Consensus Panel on Diabetic Neuropathy has emphasised the importance of integration of cardiac autonomic function testing into the current risk stratification pathways for patients with DM and established CVD risk factors[10].

The mechanisms underpinning relationship between CAN and silent ischaemia are not clear. Several mechanisms have been proposed including altered pain threshold, impaired afferent myocardial autonomic pathways and ischaemic processes not detected by routine electrocardiography. There has also been debate over whether the relationship between them is indeed a causative one, or both CAN and silent ischaemia are a product of coronary artery disease observed in diabetes[78,79].

Diabetic cardiomyopathy and LV dysfunction

Diabetic cardiomyopathy is a clinical entity that is characterised by changes in the biochemical signalling in the presence of a sympathetic-vagal imbalance resulting ultimately in left ventricular hypertrophy and remodelling, and therefore cardiac dysfunction in patients with DM in the absence of coronary artery disease[63]. Diabetic cardiomyopathy results in variable degrees of systolic and predominantly diastolic dysfunction in the absence of structural or valvular cardiac disease, coronary vessel disease, or hypertension[80,81]. Changes in the diastolic and/or systolic function can be identified on various diagnostic imaging modalities in otherwise asymptomatic patients and can precede the occurrence of macrovascular diabetic complications[82]. Frequently, the only detectable abnormality in the early stages of CAN is an isolated diastolic dysfunction with a normal LV ejection fraction[83] associated with high CVD morbidity[84,85].

Conventional echocardiography studies, with or without Doppler technique, showed that CAN is associated with significant reduction in the peak diastolic filling and an increase in the atrial component of diastole[69]. The introduction of new diagnostic modalities, such as the cardiac magnetic resonance imaging has allowed even more sensitive means of diagnosing and classifying diabetic cardiomyopathy even in the early stages by examining myocardial twist, torsion and strain[86]. Torsion is a measure of the apical rotation along the long axis of the heart and is followed by a rapid untwisting, occurring during the isovolumic relaxation phase[87]. Both torsion and maximal torsion rate have been found to be increased in patients with T2DM and preserved systolic function[86]. In patients with T1DM, increased torsion appears to be independent of energetic deficits but related to microvascular perfusion deficits and correlates with changes in sympathetic denervation[88,89]. Myocardial Perfusion Reserve (another diagnostic tool used for the detection of microvascular abnormalities) has been shown to detect the early stages of CAN in asymptomatic patients and to assess CAN severity[90].

There are several proposed mechanisms for the development of diabetic cardiomyopathy. The parasympathetic denervation observed in the early stages of the disease leads to a dominant sympathetic tone[91], which promotes a cascade of intrinsic metabolic changes, including the release of high myocardial catecholamine levels and catecholamine toxicity[92,93]. This catecholamine rise has been shown to induce mitochondrial uncoupling[94,95], switching energy generation on a cardiac level from myocardial glucose to free fatty acids, which is considered an inefficient energy source[96] and therefore increases the oxygen demand[94,95]. These alterations on the cardiac biochemical and cellular level, lead ultimately to programmed cell death and fibrosis[97,98], elevated oxygen consumption relevant to the cardiac work[99,100] and finally hypertrophy and remodelling of the LV[101]. Crucial mediators in the above process are the mitochondrial reactive oxygen species[102,103], insulin resistance[104] and calcium dependent apoptosis[102,105,106].

On a macroscopic level, diastolic dysfunction in CAN is associated with delayed relaxation, impaired filling and increased stiffness of the LV[107]. The previously described sympathetic predominance is a stimulator of the rennin-angiotensin-aldosterone axis, resulting in increased HR, cardiac output and peripheral vasoconstriction[108]. Studies have shown that this alteration on the cardiac profile can lead to reduction of coronary blood perfusion and diastolic dysfunction in patients with evidence of early microangiopathy[60]. Sympathetic overdrive may also lead LV wall stress and LV hypertrophy. Pop-Busui et al have recently shown in a large cohort of the DCCT/EDIC study, that CAN is associated with a mass increase as well as a concentric remodelling of the LV, independent of other risk factors[109].

Mortality/sudden death

CAN is associated with an increased mortality risk (Table 2). This was described in longitudinal studies in the early 1990s showing a 50% increase in 5 year-mortality risk in patients with DM and autonomic neuropathy compared to those without[110-113]. In a meta-analysis of 15 studies on the basis that they included patients with DM who had baseline assessment of HRV using one or more tests described by Maser *et al*[114] showed that the pooled estimated relative mortality risk was 2.14, (95%CI: 1.83-2.51, P < 0.0001), for those who had CAN. CAN was also found to have the strongest association with mortality amongst other risk factors in the EURODIAB IDDM Complications Study[115].

Even in patients with high CVD risk profile such as the population of the ACCORD trial, CAN was an independent predictor of all-cause mortality (HR = 2.14, 95%CI: 1.37-3.37) as well as CVD mortality (HR = 2.62, 95%CI: 1.4-4.91) after a mean follow-up of 3.5 years[29].

Table 2 Observed mortality in significant studies in the last two decades

Ref.	Country	*N* of subjects	Type of DM	FUp (yr)	Diagnostic test for CAN	Criteria applied	Mortality figures (expressed in HR, RR and incidence)	Comments
Veglio *et al*[226]	Italy	316	T1DM	5	(1) Resting heart rate (2) HRV during deep breathing (3) BP response to standing	⩾ 2 abnormal tests	Relative risk: 3.55 (1.4-8.9) and 2.21 (0.62-7.84, $P = 0.22$) after multivariate analysis for all-cause mortality	The mortality rates were 13% and 4% in the presence and absence of CAN respectively
Gerritsen *et al*[164] the Hoorn Study	Nether-lands	446	Non-DM	9	Seven parameters assessing HRV and BP response to: (1) 3-min breathing and (2) six deep breaths	Cut–off set as the lowest 25th percentile of non-diabetic group	Only E/I had a statistically significant association with mortality- Relative Risk: 2.25 (1.13–4.45) for all cause and 2.04 (0.74–5.65) for CVD mortality	An additional four parameters showed a tendency ($P < 0.10$) for association with acc-cause mortality: mean NN, LF power, HF power, and BRS
Chen *et al*[227]	Taiwan	159 612	T2DM T2DM	7.7	HRV response to: (1) single deep breath (2) six consecutive breaths (3) standing, (4) Valsalva manoeuvre	⩾ 3 abnormal tests	All cause mortality: 29% *vs* 12% with and without CAN respectively CVD mortality: 9% *vs* 2% in pts with and without CAN	The 8-yr survival rate for pts with abnormal CAN tests was 63.6% in males and 76.4% in females, compared with 80.9% and 93.3% for patients with normal CAN tests
Wheeler *et al*[228]	United States	843	T1DM and T2DM		HRV response to deep breathing and postural BP	Drop in BP ⩾ 30 mmHg and HRV divided into 5 quintiles	Hazard Ratio: 1.49 (1.01-2.19) for all-cause mortality and 1.08 (0.69-1.70) for CVD mortality	Of the 142 patients for whom cause of death was available, 75 deaths (49.7%) were due to CVD. The lowest quintile of HRV was associated with a 50% increase in mortality after adjusting for other risk factors
Astrup *et al*[229]	Denmark	388	T1DM (197 with macro-, 191 normo-albuminuria)	10.1	HRV to deep breathing	HRV < 10 bpm at baseline abnormal E/I	in the lowest quintile of HRV. Relative Risk for orthostatic hypotension: 0.65 (0.69-1.70) Relative risk: 4.9 (2.1-11.5, $P < 0.0001$) after adjustment for traditional CVD risk factors	During follow-up, 33 Patients died from cardiovascular causes, During follow-up 54 of 104 patients died: 41 patients (80.4%) with diabetic nephropathy and 13 patients (24.5%) with normoalbuminuria. Thirty patients (55%) died from cardio-vascular causes
Astrup *et al*[230]	Denmark	104	T2DM (51 with nephropathy, 52 with normal albuminuria)	9.2	HRV to deep breathing		Hazard Ratio: 0.92 (0.87–0.98, $P = 0.005$) for HRV (1 beat/min increase)	
Soedamah-Muthu *et al*[115] the EURODIAB PCS	16 European countries	2787	T1DM	7	HRV response to standing and postural BP	R-R ratio of < 1.04 and drop in systolic BP ⩾ 20 mmHg	Hazard Ratio: 3.61 (1.49–8.76) for CVD mortality and 2.83 (1.82–4.38) for all-cause mortality.	Autonomic neuropathy and microalbuminuria were the most important independent predictors of mortality
Lykke *et al*[231]	Denmark	391	T1DM	10	HRV and QTc		All cause mortality Hazard Ratio: 2.5 (0.9–6.8, $P = 0.071$) in pts with abnormal HRV and 2.3 (1.3-4.0, $P = 0.005$) in those with abnormal QT combined hazard ratio 6.7 (1.8-25, $P = 0.005$)	Out of 34 patients with both tests abnormal, 15 died in the 10 yr period (14 from cardiovascular causes)
Ziegler *et al*[232] MONICA/ KORA Augsburg Cohort study	Germany	1560 160	Non-DM DM	9	HRV, QTc interval and QTD	Group (1) Lowest quartile for SDNN, CV and max-min R-R intervals Group (2) QTc > 440, Group (3) QTD > 60 ms	All-cause mortality Relative Risk: 0.93 (0.65-1.34)/2.02 (1.29-3.17)/0.98 (0.60-1.60) in patients without DM and 1.74 (0.95-3.18)/3.00 (1.34-6.71)/0.42 (0.06-3.16) in patients with DM for group 1/2/3 respectively	Prolonged QTc interval was an independent predictor of mortality both in patients with and without DM, Low HRV trended towards an increased risk of mortality by 73% in patients with DM but not the population without DM
Beijers *et al*[233] the Hoorn Study	Nether-lands	376 114	Non-DM T2DM	13.6	HRV and BP response to: (1) 3-min breathing, (2) six deep breaths (3) standing	Calculated z-score for each parameter and averaged into a total CAD score	Relative risk: 2.54 (1.60–4.04) for CVD mortality and 2.11 (1.58–2.81) for all cause mortality,	CAN was associated with all-cause and CVD mortality independent to other CVD risk factors and microalbuminuria
Pop-Busui *et al*[29]	United States and Canada	8135	T2DM	3.5	HRV and QTI computed from 10-s resting electrocardiograms	CAN1: lowest quartile of SDNN and highest QTI quartile, CAN2: CAN1 and resting heart rate, CAN3: CAN1 and peripheral neuropathy	Hazard ratios: 1.55 (1.09-2.21)/2.14 (1.37-2.37)/2.07 (1.14-3.76) for all-cause and 1.94 (1.20-3.12)/2.62 (1.40-4.91)/2.95 (1.33-6.53) for CVD mortality in CAN1/CAN2/CAN3 respectively	CAN was independently associated with overall and CVD mortality after adjusting for baseline CVD, DM duration, traditional CVD risk factors and medications

FUP: Follow up; HR: Hazard ratio; RR: Relative risk; HRV: Heart rate variability; BP: Blood pressure; E/I: Expiration/inspiration; SDNN: Standard deviation of normally conducted R-R intervals; NN: Normal to normal R-R intervals; LF: Low frequency; HF: High frequency; BRS: Baroreflex sensitivity; CV: Coefficient of variation; QTD: QT dispersion; QTI: QT index; DM: Diabetes mellitus; T1DM: Type 1 diabetes mellitus; CAN: Cardiac autonomic neuropathy; CVD: Cardiovascular disease.

Interestingly the relationship between CAN and mortality was similar regardless of treatment allocation to the intensive or standard glycaemic control groups[29].

CAN was also found to be associated with a higher mortality risk in patients who had myocardial infarction[116], suggesting that screening for CAN in patients with DM who suffered a myocardial infarction can be used for risk stratification[117].

CAN is also associated with increased risk of sudden cardiac death[112,113,118]. This can be explained by the increased rate of fatal cardiac arrhythmias due to the imbalance between the sympathetic and parasympathetic autonomic function[119], as well as cardiac sympathetic denervation[67]. QT prolongation which has been associated with autonomic neuropathy in several studies[120-122], can also provide an alternative mechanism, rendering patients with CAN more susceptible to suffer life-threatening cardiac arrhythmias, including Torsades de Pointes[69]. The exact relationship between CAN and sudden cardiac death remains, however, under question. As shown by the Rochester Diabetic Neuropathy Study, sudden death cases are also related to severe coronary artery disease or LV dysfunction rather than CAN itself[123]. Nonetheless, as we discussed above, CAN seems to contribute to cardiovascular mortality even in those with established coronary artery disease.

Several mechanisms have been implicated in explaining the relationship between CAN and mortality in patients with DM. Autonomic neuropathy can lead to impaired response to hypoxic state[124], reduced hypoglycaemia awareness and prolonged hypoglycaemic episodes[111]. The observed mortality can also be attributed to a direct effect of autonomic neuropathy and its microvascular complications[125] as well as to an indirect association with end-organ complications, such as nephropathy, left ventricular hypertrophy and diastolic dysfunction[100]. In addition, the lack of the physiological nocturnal parasympathetic dominance in patients with CAN can lead to nocturnal hypertension, causing LV hypertrophy[126,127] and increasing the CVD burden[93,128].

Perioperative and intraoperative complications

Patients with CAN exhibit 2- to 3-times fold increase in perioperative morbidity (perioperative complications, impaired wound healing, impaired drug metabolism) and mortality[129,130]. Patients with CAN are more likely to require vasopressor support in the theatre setting[130]. They are also prone to experience a blood pressure and HR reduction during the induction of anaesthesia, as well as severe intraoperative hypothermia[131]. The above findings can be explained by an impairment or absence of the normal vasoconstrictive response to vasodilating anaesthesia in patients with CAN[130].

Cerebrovascular disease

Unlike the strong links between CAN and CVD, there is only limited data regarding the impact of CAN on cerebrovascular disease. In a study conducted by Töyry *et al*[132] that included 133 patients with T2DM, CAN was found to be an independent risk factor for developing stroke after 10 years of follow-up (OR = 6.7, 95%CI: 1.5-29.9 for HRV response to deep breathing and OR = 1.1, 95%CI: 1.01-1.2 for lying-standing BP). In a subanalysis of the Appropriate Blood Pressure Control in Diabetes population, including 950 patients with T2DM over a 5-year period, CAN was significantly associated with the occurrence of stroke, independent to other risk factors[133]. The later was also confirmed by a recent study including 1458 patients with T2DM who were followed up for 7 years[134].

Diabetic nephropathy

Several authors have hypothesized that CAN is involved in the pathogenesis of diabetic nephropathy, although causation has not been proven[135]. Sympathetic overactivity has been shown to cause glomerular and tubular dysfunction in diabetic animal models *via* indirect (hypertension and angiotensin Ⅱ) and direct (vascular smooth muscles proliferation, vasoconstriction, podocytes injury) insults[136]. CAN is associated with increased CVD morbidity and mortality[63,135] and with haemodynamic changes such as lack of nocturnal BP dipping (causing increased intra-glomerular pressure resulting in albuminuria)[137] and diurnal postural falls in BP (resulting in lower intra-glomerular pressure)[138] and endothelial dysfunction in humans. In addition, CAN is associated with deficits in erythropoietin production and, as a result, erythropoietin-deficiency anaemia[137]. Subsequently, CAN patients are deprived from the direct nephroprotective action of erythropoietin and thus, anaemia becomes a strong predictor of nephropathy and progression of chronic kidney disease[68]. In streptozotocin-diabetic rats, sympathetic overactivation has been shown to be involved in the pathogenesis of diabetic nephropathy[139] and renal denervation was shown to prevent glomerular hyperfiltration[140]. Hence it is plausible that CAN is involved in the development and progression of diabetic nephropathy. Several studies examined the association between CAN and either albuminuria and/or glomerular filtration rate[141-145], but all these studies had a cross-sectional design, hence causation cannot be proven, particularly that the pathogenesis of CAN is similar to other microvascular complications including diabetic nephropathy. Longitudinal studies are scarce and limited to a small number of patients with T1DM[138,146]. Hence, data regarding the longitudinal impact of CAN on diabetic nephropathy in patients with T2DM is lacking.

Lower limb complications

CAN has been proposed as a contributing factor in the development of lower limb vascular and neurological complications. Autonomic neuropathy can cause alterations in microvascular blood flow (MBF), which predispose to changes in skin structure and quality and impairment of sweat glands' innervation resulting in dry skin and increased risk of oedema and foot deformity which increases pressure on certain areas causing ulceration[147]. It is also believed that CAN, through the sympathetic

denervation of the lower limb vasculature, can induce lower extremity hyperaemia, increase inflammation and erosion into the joints/bones and therefore contribute in Charcot's neuroarthropathy. As a result, the patient with Charcot will typically present with prominent peripheral pulses due to vasodilatation and autonomic neuropathy. Power Spectral Analysis and HRV has been employed in trials for the detection of autonomic neuropathy in patients with Charcot's disease[148]. Similarly to Charcot's arthropathy, patients with recurrent vascular neuropathic ulcers appear to share analogous cardiac autonomic dysfunction, as shown by the use of HRV, Valsalva ratio and orthostatic hypotension[149].

THE DIAGNOSIS OF CAN

CARTs

Ewing *et al*[150] proposed in early 1970s five simple non-invasive tests to measure cardiac autonomic function based on the HR and blood pressure response to certain physiological manoeuvres. These tests include: (1) the HR response to deep breathing, which assesses beat to beat HR variation (R-R variation) during paced deep breathing [expiration-to-inspiration ratio (E:I)]; (2) the HR response to standing, which is expressed as the 30:15 ratio which is the ratio of the longest R-R interval (between the 20th and 40th beat) to the shortest R-R interval (between beats 5-25) elicited by a change from horizontal to vertical position; (3) the Valsalva manoeuvre which evaluates the HR response during and after a provoked increase in the intra-thoracic and intra-abdominal pressures (the patient normally exhales for a period of 15 seconds against a fixed resistance); (4) the blood pressure response to standing, which assesses the baro-reflex mediated blood pressure change following postural change; and finally; and (5) the blood pressure response to sustained handgrip, as defined by the diastolic blood pressure increase caused by the sustained muscle contraction with the use of a handgrip dynamometer[17]. The first two tests reflect defects in the parasympathetic activity (*i.e.,* the ability of the vagal nerve to slow the HR during the procedures which increases the R-R interval and hence increases the ratios), while the last two also describe changes in the sympathetic function (*i.e.*, the ability to provide appropriate BP and HR response to the activity involved)[151,152]. The autonomic changes that occur during the Valsalva manoeuvre are complex and involve both the sympathetic and parasympathetic systems[153], although the Valsalva ratio mostly represents parasympathetic activity. For more details about the autonomic changes during Valsalva please see[17].

While the above described CARTs have been widely used since their introduction, there is no evidence on the superiority of one test over another when it comes to assessing CAN[10]. However, the HR response to deep breathing is the most commonly utilised, because of its high reproducibility and specificity[154] and its ease of use[10,155]. All the tests are considered to be valid markers of autonomic dysfunction, given that end organ failure is excluded and parameters such as concomitant illness, use of over the counter medications and lifestyle factors (exercise, smoking, exercise) are taken into consideration[156].

HRV

A reduction in HRV has been associated with the early stages of clinical CAN. In healthy individuals, the beat-to-beat variability with aspiration is predominantly affected by the direct sympathetic and parasympathetic activity[62,157], as well as various other stimuli, including certain neurohumoral factors (catecholamines, thyroid hormones), temperature changes and mechanical and ionic changes in the sinus node[158]. The efferent sympathetic and vagal stimulation is characterised by synchronised discharges, modulated by central and peripheral oscillators, with the former referring to vasomotor and respiratory centres and the later to respiratory movements and arterial pressure. These synchronous neural discharges can manifest as short and long -term oscillations in the HR[63].

The R-R intervals recorded under paced breathing are transformed to generate the time and frequency domains. Conceptually, if the faster respiratory sinus arrhythmia signal and the slower mean HR changes could each be separated from the patient's cardiogram and analyzed independently, the result would yield a measure of Vagal outflow from the respiratory sinus arrhythmia and a measure of sympathetic activity from the changes in mean HR. Effectively this is what is accomplished in the frequency- or spectral-domain. Spectral analysis of respiratory sinus arrhythmia provides the indication of where in the frequency domain the Vagus is influencing the heart. The frequency domains are generated using continuous wavelet transform method (Fourier transform) and separated to three basic components: very-low-frequency, low-frequency (LF) and high-frequency (HF)[61,159]. HF represents vagal activity, whereas LF is attributed to the combined effect of sympathetic and parasympathetic influence[62,160]. Modern software (such as ANSAR technology) adjusts for the respiratory rate, hence simplifying the process. Parameters generated include: respiratory frequency (Rfa, 0.15 to 0.4 Hz, represents parasympathetic function), and LF (Lfa, 0.04 to 0.15 Hz, represents sympathetic function). The HRV and BP are recorded with the patient in sitting position during resting, deep breathing, Valsalva manoeuvre and standing position.

The electrocardiogram (ECG) recordings were initially longer in duration, usually over a period of 24 h but recent data has demonstrated that recording of shorter duration can provide equally reliable information[16,158,161]. Time domain analysis is a useful tool in the assessment of parasympathetic activity by measuring the normal R-R intervals, whereas the frequency domain is based on the spectral analysis of R-R interval and other cardiovascular and respiratory signals based on short-term ECG recordings (2-5 min)[69,158].

The key element in the accurate use and interpretation of HRV models is the standardisation of the conditions under which the test is carried, including age, blood pres-

sure, HR, tobacco smoking or caffeine use and, above all, respiration control[69].

Baro-reflex sensitivity

The BRS measures the cardiac vagal and sympathetic baro-reflex function. The idea behind its function is that an increase in the BP normally induces a reflective increase in the vagal cardiac efferents and a reduction to the efferent sympathetic activity, resulting in bradycardia and hypotension, due to the reduction in cardiac output as well as the peripheral vasodilation[158]. A reduction in BP induces opposite responses. Thus, to correctly measure the baro-reflex function, both the vagal efferent activity (evidenced by changes in HR in response to changes in BP), and the sympathetic efferent activity (affecting the arterial vessels) should be taken into account.

In practice, the term "baro-reflex sensitivity" normally applies to the cardiac-vagal arm, and to methods measuring changes in HR in response to changes in (systolic) BP. The test can be performed either with the use of pharmacological methods (intravenous bolus injection of epinephrine)[162] or non-pharmacological techniques (physical manoeuvres such as postural change). Although the former is considered the gold standard to date for evaluating BRS, both of them correlate well clinically with each other[163]. Both techniques require a continuous measure of BP and a continuous and synchronised measure of HR (R-R interval)[158].

BRS can be used for detecting sub-clinical CAN[63], since BRS can be abnormal in diabetes, before the demonstration of any clinical signs of CAN or other conventional autonomic function tests detect any abnormalities[64,65]. Several studies on patients with diabetes have concluded that BRS is a strong independent risk factor for mortality[164], especially in cohorts suffering from heart failure or following a myocardial infarction[162,165].

Scintigraphy

The use of Single-photon emission computed tomography (SPECT) and/or positron emission tomography (PET) and sympathetic neurotransmitter analogues, such as the ^{123}I-metaiodobenylguanide (^{123}I-MIBG) (SPECT), the ^{11}C-metahydroxyephedrine (^{11}C-HED) (PET) and ^{11}C-epinephrine has enabled the quantitative scintigraphic evaluation of cardiac sympathetic innervation[63].

^{123}I-MIBG undergoes rapid uptake in the myocardium but as it is semi-quantitative is not a precise indicator of neuronal uptake[158]. Metabolically stable ^{11}C-HED demonstrates a highly specific uptake by the sympathetic nerves mediated by norepinephrine transporters[166]. It is important, however, to take myocardial perfusion (which affects the delivery of the tracer of interest) into consideration before interpreting the results of these imaging techniques. Retention defects of both ^{123}I-MIBG and ^{11}C-HED have been reported in patients with T1DM and T2DM and have been variably correlated with abnormal but also normal CARTs[60,67,167]. The consistent pattern of sympathetic denervation in patients with T1DM supports the notion that ^{11}C-HED can be used to monitor the population of sympathetic nerves and evaluate the regional autonomic deficits of sympathetic innervations[66,166,167]. In patients with CAN and T1DM, the wash rates of ^{11}C-epinephrine have been shown to correlate well with those of ^{11}C-HED[158]. The development of microvascular complications has been associated with the augmentation in sympathetic tone and adrenergic hyperresponsiveness, by the use of ^{11}C-HED[63]. As CAN reaches an advanced stage, a heterogenous pattern of ^{11}C-HED retention is observed, with a reduced ^{11}C-HED retention in the distal LV and a persistent or increased ^{11}C-HED retention seen proximally, indicating a proximal to distal pattern of sympathetic denervation of the LV[63].

Increases in the sympathetic nervous tone and elevated epinephrine levels can affect the retention of sympathetic neurotransmitter analogues, making the interpretation of the above scintigraphic models rather challenging. Furthermore, the lack of standardisation, the high cost and the demand on highly skilled operators, restricts the role of scintigraphy as a valuable research tool and not a part of daily clinical routine[68].

When it comes to radiation exposure, ^{123}I-MIBG lacks a β-particle emission and has a half-life of 13.2 h, whereas its energy of the primary imaging photon is calculated at 159 keV (kiloelectron volt)[168]. When compared to ^{131}I, ^{123}I-labelled agent is to be considered the radiopharmaceutical of choice as it has a more favourable dosimetry and better radiation profile. Whole-body radiation is markedly lower using ^{11}C-HED PET [effective dose equivalent in adults, 1.2 milliSieverts (mSv)] compared with ^{123}I-MIBG scintigraphy (effective dose equivalent in adults, 6.0 mSv)[169]. The radiation dose to the whole body from 20 milliCuri (mCi) ^{11}C-HED is 0.186 rad, less than that from 0.5 mCi ^{131}I-MIBG IBG (0.45 rad) or 10 mCi ^{123}I-MIBG (0.53 rad)[170].

Muscle nerve sympathetic nerve activity

Muscle sympathetic nerve activity (MSNA) is based on the ability to record efferent sympathetic nerve signals in the skeletal muscles either at rest or in response to physiological perturbations with the use of microelectrodes into a fascicle or a distal sympathetic nerve of the skin or muscle (microneurography)-usually the peroneal nerve[171].

MSNA is the most direct measure of peripheral sympathetic activity and therefore a useful research tool. However, its invasiveness, cost and time-consuming nature is not recommended for routine autonomic assessment[158].

Other tests

Occasionally, various tests have been proposed for the assessment, diagnosis and monitoring of CAN. A recent study on 167 patients with type I diabetes conducted by the University of Liege, found the use of pulsatile stress, which measures the arterial stiffness, correlates well with baro-reflex sensitivity, suggesting therefore that arterial stiffness can be used as a marker of CAN[172]. The association between arterial stiffness (expressed as carotid-femoral wave velocity (PWV)) had already been explored

Significance of CAN

According to the Toronto Consensus Panel on Diabetic Neuropathy statement, *screening for CAN* in the patients with DM should be considered good clinical practice, due to the following:

(1) It enables the accurate and clinical relevant diagnosis of various CAN forms

(2) It assists in the appropriate detection and subsequently the tailored treatment of CAN multiple clinical manifestations as described in the previous section

(3) It provides a clinical tool for the risk stratification for diabetic complications as well as the cardiovascular morbidity and mortality

(4) It can be used for the modulation of targets of diabetes treatment

Figure 3 Current recommendations on screening for cardiac autonomic neuropathy. CAN: Cardiac autonomic neuropathy; DM: Diabetes mellitus.

by another study. After multivariable linear regression, the association between CAN (E/I index in particular) and PWV not only remained significant but E/I index was the strongest predictor of PWV in the model (β coefficient: -0.326, 95%CI: (-3.110)-(-0.750), $P = 0.002$)[173]. Catecholamine kinetics, most specifically epinephrine and norepinephrine plasma clearance have been labelled as the biochemical equivalent of MSNA but they have failed to date to produce reliable diagnostic data[158].

Another aspect of autonomic function is the assessment of cutaneous MBF. The skin offer an accessible organ to asses MBF and endothelial function, which is often involved in the development of micro and macrovascular diabetes, correlates with systematic endothelial function measures and myocardial microcirculation[174]. Several methods are available to assess skin MBF[175]. Laser Doppler (LD) allows the determination of blood flow under basal conditions or following physical (*e.g.,* heating) or pharmacological (*e.g.,* acetylcholine and/or sodium nitroprusside) stimulation; allowing the differentiation between endothelial-dependant and independent responses[174]. Furthermore, LD allows the measurement of nerve axon reflex-related vasodilation following acetylcholine iontophoresis which is the result of C-fibre stimulation[176]. LD techniques include LD flowmetry, LD perfusion imaging and laser speckle contrast imaging[158,174].

Another assessment of the peripheral autonomic system is intra epidermal nerve fibre density (IENFD) using immuno staining[177]. IENFD is highly sensitive and specific to diagnose small fibre neuropathy (88%-98% and 88.8%-95% respectively)[178]. IENFD correlates also inversely with thermal thresholds[178]. In addition, IENFD innervates the sweat glands. Reduction in sweat production in the feet contributes to the development of dry skin/callus and hence predispose to the development of foot ulceration. This function can be assessed by several methods such as Neuropad[147] and Sudoscan[179].

CRITERIA FOR DIAGNOSIS AND STAGING

HR responses to deep breathing, standing and Valsalva manoeuvre, as well as blood pressure response to standing (CART) are considered as the gold standard in clinical testing for autonomic neuropathy[10]. Their applicability in bedside clinical practice is based on their sensitivity, specificity, reproducibility, ease and safety of use and standardisation.

According to the CAN Subcommittee of the Toronto Consensus Panel statement following the 8th international symposium on diabetic neuropathy in 2010[10], the criteria for diagnosis and staging of CAN are as follows: (1) A single abnormal CART result suffices for the diagnosis of possible or early CAN; (2) The presence of two or three abnormal test among the seven autonomic cardiovascular indices (5 CARTS, time-domain and frequency-domain HRV tests) are required for the diagnosis of definite or confirmed CAN; and (3) The presence of orthostatic hypotension in addition to the above criteria signifies the presence of severe of advanced CAN.

SCREENING FOR CAN

The majority of diabetes patients with CAN have subclinical or asymptomatic disease, rendering the diagnosis and appreciation of CAN in clinical practice rather difficult[63]. Once CAN reaches the stage that becomes clinically evident, the disease might have reached an advanced level and management becomes more difficult. Screening for early CAN is therefore considered good clinical practice several reasons as summarised in Figure 3[10].

The Toronto Diabetic Neuropathy Expert Group in a recent statement have recommended that screening should be considered for patients at time of diagnosis of T2DM and within 5 years of diagnosis of T1DM, particularly in patients with other macro- and/or microvascular complications[180]. Patients with a history of poor glycaemic control are especially at risk for developing CAN, as demonstrated in several studies, suggesting that this clinical group may benefit from screening[17]. Due to its impact on exercise tolerance, testing for CAN should be a part of the screening in patients that are about to begin a new exercise programme that involves more intense physical activity than brisk walking[69,181]. Evidence also suggests that screening for CAN could be incorporated into the perioperative assessment of patients with poor glycaemic control and coronary artery disease, due to the association between CAN and haemodynamic instability peri- and intra-operatively[182]. Finally, testing for CAN could potentially be of benefit in patients with DM that have suffered MI, as this would serve in the risk stratifica-

tion of this subgroup and assist into adapting a more aggressive therapeutic approach for those at risk of sudden cardiac death or life threatening arrhythmias.

THERAPEUTIC APPROACHES FOR CAN

CAN treatment can either be symptomatic or aimed at slowing or reversing CAN progression. However, effective therapies to slow or reverse CAN progression are rather limited as the complete underlying pathogenesis remains unclear. However, based on our current understanding of CAN pathogenesis and risk factors, several potential treatments have been examined.

Lifestyle modification

Lifestyle changes have been shown to have a beneficial impact on the prevention of CAN progression in the Steno-2 trial[5] and the Diabetes Prevention Program (DPP)[183]. In the Steno-2 study, patients with T2DM and microalbuminuria were randomised to a multi-factorial cardiovascular risk factor intervention that included behavioural therapy (diet, physical exercise and smoking cessation) and pharmacological intervention (to control BP, lipids and hyperglycaemia) or conventional treatment in accordance to the national guidelines. After an average of 7.8 years of follow-up, the risk for developing CAN was significantly lower on the intervention arm (49% in the intensive group *vs* 65% in the conventional group, HR = 0.37, 95%CI: 0.18-0.79, $P = 0.002$). In the DPP, lifestyle modification demonstrated superior results in the improvement of autonomic dysfunction (assessed with HRV and QT indexes) as compared to the use of metformin or placebo.

Weight loss and dietary intervention accompanied[69] or not[184] by supervised training was associated with improvement on CAN indices. Aerobic training has also been shown to improve CAN, with some indication that mild physical exercise is recommended in less severe CAN cases. A recent review summarising the evidence for the impact of life style interventions on CAN has concluded that moderate endurance and aerobic exercise in both T1DM and T2DM, improve HRV and cardiac autonomic function significantly, in favour of parasympathetic dominance, independent of BMI, glycaemic or BP control and duration of diabetes[185].

Intensive glycaemic control

Hyperglycaemia is a major risk factor for CAN development and progression. Intensive glycaemic control has been shown to slow the progression and prevent/delay the development of CAN[18,66,186,187]. In the DCCT trial, intensive glycaemic control in a group of patients with T1DM reduced the CAN incidence by 50% over 6.5 years follow-up compared with conventional therapy (7% *vs* 14% respectively)[19]. These beneficial effects persisted 13-14 years after close-out of the trial[18]. Although both former treatment arms exhibited deterioration in CAN during follow-up after the end of the DCCT, the former intensive treatment group continued to demonstrate a statistically significant slower decline in CAN.

PET cardiac imaging with the use of 11C-HED showed similar beneficial effects in a 3-year prospective trial. Good glycaemic control (defined as mean HbA1c < 8%) was associated with reduction of sympathetic denervation as opposed to the group of poor diabetes control (HbA1c $\geqslant$ 8%)[167]. In the SEARCH CVD study, 354 young patients with T1DM were assessed for the presence of sub-clinical autonomic dysfunction, as demonstrated by the use of HRV parameters and the presence parasympathetic loss with sympathetic override. Poor glycaemic control, as defined by HbA1C > 7.5%, was independently associated with the presence of subclinical CAN as compared to a frequency-matched control group without DM[188].

The effects of glycaemic control in T2DM are not similarly encouraging. Data from recent studies have failed to demonstrate differences in the incidence of CAN based on the application of intensive therapy in T2DM patients[189,190]. The sensitivity of tests utilised for the diagnosis of CAN in those trials, however have been questioned, suggesting that more research is needed to investigate the relationship between metabolic control and CAN in patients with T2DM.

Therapies based on CAN pathogenesis

There is limited but increasing data on the use of pharmacotherapy targeting specific pathogenic pathways. The use of the specific antioxidant α-lipoic acid improved CAN in patients with T2DM in a 4-mo controlled randomised trial[191]. In animal models, the pharmacological agents FP15 and FeTMPS, which act by catalysing the decomposition of peroxynitrite, have shown promising results in improving neuronal function[192-194]. The use of glucagon-like peptide 1 analogues or the dipeptidyl peptidase 4 inhibitors have demonstrated cardioprotective[195] and neuroprotective properties[196], raising the possibility of their use for treatment not only for peripheral neuropathy, but autonomic neuropathy as well. In small scale studies, aldose reductase inhibitors have been shown to improve LV function in patients with DAN without any alteration on CAN indices[197]. There is also evidence suggesting the vitamin E and C-peptide can both improve HRV indices[10]. In a randomised controlled trial, vitamin E when compared to placebo managed to increase the R-R interval ($P < 0.05$) and the HF component of HRV (HF; $P < 0.05$) in 50 patients with T2DM over a period of 4 mo[198]. Small RCTs have shown beneficial effect of C-peptide treatment on CAN parameters[53]. In a recent randomised placebo-controlled trial of 44 patients with T1DM, treatment with a triple antioxidant regime (allopurinol, α-lipoic acid and nicotinamide) over the course of 2 years failed to prevent progression of CAN and had no benefit on myocardial perfusion as demonstrated with scintigraphic imaging modalities[199]. Further research is required to confirm these findings and explore other potential pathogenetic therapies.

The renin-angiotensin-aldosterone axis

There is substantial data to support the use of certain pharmacological agents in the improvement of the left ventricular dysfunction associated with autonomic neuropathy in diabetes. In patients with heart failure, the use of bisoprolol[200] or the addition of spironolactone to enalapril, furosemide and digoxin[201], demonstrated a beneficial effect on autonomic function, as shown by HRV testing and sympatho-vagal balance respectively. The use of angiotensin-converting enzyme (ACE) inhibitors could potentially improve the parasympathetic/sympathetic balance[202] and improve prognosis in cardiac failure[203]. The addition of angiotensin receptor blockers to ACE inhibitors may be superior to monotherapy[204-206], due to the enhanced blockade on the renin-angiotensin-aldosterone axis[207]. In a small study by Didangelos *et al*[208], including 62 patients with type I and type II DM, the use of ACE inhibitors or ARBs, as well their combination, managed to improve both diabetic autonomic neuropathy and LV diastolic dysfunction.

Symptomatic treatment of orthostatic hypotension

Treatment of orthostatic hypotension is required in symptomatic patients with autonomic neuropathy. There are several strategies available, including lifestyle and behavioural measures as well as pharmacological options. The former include advice provided to the patients to avoid sudden changes in body posture, eat smaller and more frequent meals, avoid drugs-precipitants of postural hypotension (diuretics, tricyclic antidepressants, α-adrenoreceptor antagonists), perform physical countermanoeuvres (leg crossing, stooping and squatting), increase fluid and salt intake, avoid physical activity that leads to straining and finally use garments over legs and abdomen[69,209].

If the above measures fail to improve symptoms, pharmacological intervention may be considered. A risk-benefit consideration should take place for each individual before starting a medication, especially weighing up the risk of developing marked supine hypertension against the benefit of preserving the erect blood pressure. Should a pharmacological agent be considered appropriate by the clinician, there are several options available[210-212].

Midodrine, a peripheral selective α_1-adrenergic agonist, is considered a first line agent that acts through peripheral vasoconstriction of arterioles and veins. It remains to date the only drug approved by the food and drug administration (FDA) for the treatment of orthostatic hypotension[213,214]. However, post-market trials to prove drug's efficacy are still ongoing and the final results on midodrine's benefits are scheduled to be published in 2014, 18 years after the drug was given FDA approval[215].

9-α-fluorohydrocortisone, a synthetic mineralocorticoid, is another first line option that acts through sodium retention and plasma expansion[216]. In a double-blinded crossover study, 9-α-fluorohydrocortisone treated successfully the orthostatic hypotension of patients with diabetes and autonomic neuropathy[216]. 9-α-fluorohydrocortisone doses between 100 and 400 micrograms decreased significantly the orthostatic hypotension in 14 symptomatic patients with DM over a mean period of 12 mo ($P < 0.001$)[217]. Extra care should be taken when prescribed in patients with cardiac failure, as it can lead to fluid overload. There is usually a period of 10-14 d before its effects can become clinically evident[212].

Somatostatin and somatostatin analogues (octreotide) inhibit the release of vasoactive peptides from the GI tract and thus increase splanchnic vasoconstriction, leading to increase in mean blood pressure[218]. The use of long acting octreotide in patients with autonomic neuropathy increased the mean systolic BP from 83.8 ± 7.1 mmHg to 104.1 ± 3.1 mmHg ($P < 0.025$) within eight weeks, improving orthostatic dizziness and fatigue[219]. In a study of 18 patients with idiopathic orthostatic hypotension, octreotide reduced postural, postprandial and exertion-induced hypotension, as demonstrated by 24-h ambulatory blood pressure profiles and cusum analyses[220].

Other available pharmacological strategies include the use of erythropoietin which can increase the erect BP through the increase of red cell mass and circulating volume, the improvement of anaemia and its regulatory effect on vascular tone[221] and desmopressin acetate whose efficacy is mainly observed in morning time hypotension[212]. Finally, caffeine and acarbose can potentially be used in the management of post-prandial hypotension[212]. In a case report of 58 years old patient with DM and severe postprandial hypotension refractory to the use of midodrine and octreotide, acarbose (an alpha-glucosidase inhibitor) reduced the postural drop from 50 mmHg to 18 mmHg, improving the patients symptoms dramatically[222].

Unfortunately, despite the different options available, postural hypotension remains a difficult condition to treat and many patients require multiple therapies and develop severe intractable disabling symptoms. Beta blockers might help controlling the tachycardia in some patients[69].

CONCLUSION -SYNOPSIS AND FUTURE CONSIDERATIONS

CAN is very common and is an underdiagnosed complication of DM. CAN is associated with significant increase in morbidity and mortality and plays an important role in the development of diabetic cardiomyopathy and silent ischaemia. Clinicians interpreting exercise tolerance testing should be aware of the reduced accuracy of this test in patients with CAN. In addition, CAN might play a role in the pathogenesis of diabetes-related microvascular complications and the development of lower limb complications. However, before CAN is symptomatic and evident clinically, patients might have sub-clinical CAN for several years. The time scale for the progression from sub-clinical to clinically evident CAN is unknown. In addition, the time scale for the progression from early abnormalities (such as increased LV torsion) to clini-

cally detectable cardiac disease is also unknown. Recent guidelines have recommended screening for CAN in patients with diabetes and issued guidance regarding the criteria used to diagnose CAN. CAN is assessed using several methods including CARTs, HRV, and imaging amongst others. The use of HRV and spectral analysis has simplified CAN testing which nonetheless remains time consuming. Despite our improved understanding of the pathogenesis of CAN, disease modifying treatment is lacking. Improving glycaemic control, life style changes and CVD risk factors management are the mainstay of treatment, which generally slow the progression of CAN rather than reversing it.

Further research exploring the natural history of CAN and the natural history of the impact of CAN on CVD is needed. Better understanding of CAN pathogenesis is also required in order to develop disease modifying treatments. OSA is increasingly recognised as an important contributor to the development of microvascular complications in DM, hence it is important to clarify the relationship between CAN and OSA as this might identify new treatment targets.

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REVIEW

Insulin plus incretin: A glucose-lowering strategy for type 2-diabetes

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Abstract

There are many advantages of combining incretin therapy [glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors] with insulin therapy as a glucose-lowering strategy in type 2 diabetes. One important advantage is the complementary mode of the mechanistic action of incretin and insulin therapy. Another advantage is the reduction in risk of hypoglycemia and weight gain when adding incretin therapy to insulin. Several clinical trials have studied the addition of GLP-1 receptor agonists [exenatide BID (twice daily), lixisenatide, albiglutide] or DPP-4 inhibitors (vildagliptin, sitagliptin, saxagliptin, alogliptin, linagliptin) to ongoing insulin therapy or adding insulin to ongoing therapy with a GLP-1 receptor agonist (liraglutide). These studies show improved glycemia in the presence of limited risk for hypoglycemia and weight gain with the combination of incretin therapy with insulin. This article reviews the background and clinical studies on this combination.

Key words: Type 2 diabetes; Glucose lowering; Insulin therapy; Glucagon-like peptide-1 receptor agonists; Dipeptidyl peptidase-4 inhibitors; Incretin therapy; Combination

Core tip: Incretin therapy (glucagon-like peptide-1 receptor agonists or dipeptidyl peptidase-4 inhibitors) combined with insulin therapy is a glucose-lowering strategy in type 2 diabetes. The combination allows a complementary mode of mechanistic action and, as demonstrated in several clinical trials, is glucose-lowering in association with limited risk for hypoglycemia and weight gain. The combination is a promising strategy in patients in whom metformin with either incretin therapy or basal insulin is insufficient for adequate glycemic control. This article reviews the background and clinical studies on this combination.

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INSULIN IN COMBINATION WITH INCRETINS: A MORE COMMONLY USED GLUCOSE-LOWERING THERAPY

Life style changes accompanied by addition of metformin are often first line glucose reducing therapy in type 2 diabetes[1,2]. When metformin as the only pharmaceutical agent is insufficient for adequate glycemic control, several options are currently available. Of these, sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists and insulin were recently suggested by the joint position statement from the American Diabetes Association and the European Association of the Study of Diabetes to be potentials as an add-on to metformin[1]. They were suggested to be individualized to target the best combination for the individual patient. However, even after combination of metformin with any of these second-line thera-

pies, many patients still do not reach the glycemic target which is mainly due to the progression of the disease. At this stage, three-drug combinations are suggested to be used, involving metformin in combination with two of the other options. One such three-drug combination is the combination of insulin therapy with incretin therapy (+ metformin) as a glucose-reducing strategy of type 2 diabetes[2-6]. This article reviews the current evidence and experience for this combination.

BASIS FOR INCRETIN THERAPY

Incretin therapy is based on the anti-diabetic effects of GLP-1[7]. As an incretin hormone, GLP-1 is released from the gut after meal ingestion and augments insulin secretion in a glucose-dependent manner[7,8]. This effect on the beta cells is achieved through activating specific GLP-1 receptors, which are G protein coupled receptors[9]. GLP-1 also has an important effect to inhibit glucagon secretion[10]. These double effects on islet hormone secretion are of importance for the anti-diabetic action of incretin therapy and, furthermore, by targeting the double alpha and beta cell dysfunction, incretin therapy targets a main pathophysiological cause of the disease[11]. GLP-1 receptors are, however, also expressed in other cells and therefore GLP-1 also exhibits extra-islet effects, such as delay of gastric emptying[12] and satiety through a central effect in the hypothalamus[13]. GLP-1 also has the potential of preserving beta cell function through inhibition of apoptosis[14], although this has so far only been demonstrated in animal studies and not shown in humans.

The first study showing an anti-diabetic action of GLP-1 was published in 1992[15]. In the early development of GLP-1 as a therapy, GLP-1 had to be given as an intravenous infusion since the hormone is rapidly inactivated by DPP-4[16]. The two successful strategies for incretin therapy used this knowledge and today we have several GLP-1 receptor agonists which are not or only weakly inactivated by DPP-4 and DPP-4 inhibitors[17-20].

GLP-1 receptor agonists are injected subcutaneously once or twice daily [exenatide BID (twice daily), liraglutide, lixisenatide] or once weekly {exenatide once weekly [Quaque weekly (QW)]}. In addition, once weekly GLP-1 receptor agonists are in late clinical development (albiglutide, semaglutide, dulaglutide)[17,20]. The GLP-1 receptor agonists therefore differ in several respects, such as dosage regimen. However, GLP-1 receptor agonists also differ in other aspects, as was recently reviewed[17-20]. Thus, the different GLP-1 receptor agonists have different molecular structures and in this context, they may be derived from exendin-4, showing approximately 50% homology with native GLP-1 (exenatide, lixisenatide), or they may be true GLP-1 analogues with a structure showing a high (> 90%) homology to GLP-1 (liraglutide, albiglutide, semaglutide, dulaglutide). The GLP-1 receptor agonists also differ in molecular size since they may be similar in size to native GLP-1 (exenatide, lixisenatide, liraglutide, semaglutide) or be 15-20 times bigger because of fusion of GLP-1 with albumin (albiglutide) or immunoglobulin (dulaglutide).

DPP-4 inhibitors are oral agents given once or twice daily (sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin, tenelagliptin, anagliptin, gemagliptin)[18,19]. They are different from each other in terms of molecular structure, although they are all small molecules, and they also differ, besides in pharmacokinetics with relevance for dosing regimen, in elimination mechanisms, as was recently reviewed[21].

Incretin therapy is today established as an add-on treatment to metformin and is also used in other conditions; it results in reduction of both fasting and postprandial glucose and it is associated with a low risk of hypoglycemia and no weight gain (weight reduction or weight neutrality)[19,20,22].

RATIONALE FOR COMBINATION INSULIN THERAPY PLUS INCRETIN THERAPY

The combination of incretin therapy and insulin therapy was initially not clearly evident during the development of incretin therapy. Instead, incretin therapy was mainly developed for combination with oral antihyperglycemic agents, in particular metformin. This is still a very important combination. However, as discussed for GLP-1 receptor agonists[4] and DPP-4 inhibitors[2,3], incretin therapy offers mechanistic advantages when used in association with insulin, which makes this combination a promising strategy for treatment.

The mechanistic complementary actions of the two approaches relate to reduction in fasting glucose, reduction in postprandial glucose, the low risk for hypoglycemia and the prevention of weight gain. More mechanistic studies are required, however, for a full appreciation of the complementary actions of insulin and incretins in combination.

Fasting glucose

Reduction of fasting glucose is a major goal for glucose-lowering therapy since fasting glucose contributes largely to hemoglobin A1c (HbA1c)[23,24]. A main effect of basal insulin is the reduction of fasting glucose, which is achieved through increased peripheral (mainly muscle and fat tissue) glucose utilization and inhibited hepatic glucose output[25,26]. Also, GLP-1-receptor agonists and DPP-4 inhibitors reduce fasting glucose but this is achieved through other mechanisms than insulin; mainly a glucose-dependent inhibition of glucagon secretion from the islet alpha cells[10,27]. In addition, direct liver effects of GLP-1 may also contribute[28]. Hence, the combination of insulin with incretin therapy would be expected to complement each other to reduce fasting glucose.

Postprandial glucose

Postprandial glucose also contributes to HbA1c and is therefore a target for glucose-lowering therapy[23,24]. Postprandial glucose is mainly regulated by gastric emptying

and the meal-induced islet hormone responses[29-31]. These effects are not appreciably affected by basal insulin. In contrast, incretin therapy reduces postprandial glucose, although the mode of action to achieve this effect differs between GLP-1 receptor agonists and DPP-4 inhibitors. GLP-1 receptor agonists reduce postprandial glucose mainly by delaying gastric emptying[29-31]. This effect of GLP-1 shows, however, tachyphylaxis, meaning that during long-term and continuous stimulation, the effect is reduced[32,33]. Consequently, intermittently acting GLP-1 receptor agonists (exenatide BID, lixisenatide) have been shown to be more potent to reduce gastric emptying than continuously acting GLP-1 receptor agonists (liraglutide, exenatide QW)[34,35]. In contrast, DPP-4 inhibitors do not inhibit gastric emptying[36] but instead reduce postprandial glucose mainly through inhibiting postprandial glucagon levels and stimulating beta cell function[27,37]. Both incretin therapy strategies therefore reduce postprandial glucose and thus complement the lack of such an effect by insulin in the combination therapy.

Hypoglycemia

Hypoglycemia is an adverse event for glucose-lowering therapy and is occasionally the limitation factor for achieving good glycemic control. Hypoglycemia is associated with negative impact, such as unpleasant and sometimes dangerous symptoms, weight gain (due to defense eating), deterioration of glycemic control (due to reduced adherence to therapy and therapeutic goals because of fear of new hypoglycemic episodes), increased cardiovascular risk and increased risk for microvascular complications[38-41]. Insulin therapy is associated with a high risk of hypoglycemia[29-31]. In contrast, incretin therapy is associated with a low risk of hypoglycemia[30,31,39-47]. This is because the islet effect of GLP-1 is glucose dependent[7,9] and the glucagon counter-regulation to hypoglycemia is preserved or augmented[48-50]. Therefore, incretin therapy has the potential to prevent the hypoglycemia induced by insulin when the two treatments are used in combination.

Body weight

Since increased body weight is associated with long-term negative effects, prevention of weight gain or weight reduction is of importance for glucose-lowering therapies. Body weight is increased by insulin therapy[51]. This is due to the anabolic action of insulin but may also be due to the self-defense eating associated with hypoglycemic events. Incretin therapy, on the other hand, prevents weight gain since its lowering of glycemia is not associated with increased risk of hypoglycemia and therefore the therapy avoid the self-defense eating[52]. GLP-1 receptor agonists also induce satiety through effects on the satiety center in the hypothalamus, thereby inducing weight reduction[13]. Therefore, the combination of incretin therapy with insulin has a great advantage of preventing the weight gain induced by insulin.

Disease modifying effects

Type 2 diabetes is a progressive disease with is mainly due to a continuous decline in beta cell function[53]. It has been discussed whether insulin therapy and incretin therapy may have complementary disease modifying effects[5]. The rationale for this suggestion is that insulin has been suggested to improve beta cell function through its normalization of fasting glucose, thereby preventing glucotoxicity and may also result in "beta cell rest"[54]. On the other hand, GLP-1 based therapies may improve beta cell function so much that beta cell function will also be improved over a long-term perspective, particularly in association with inhibited beta cell apoptosis[7,9].

ADVANTAGES OF COMBINING INSULIN WITH INCRETIN THERAPY

The complementary actions of insulin and incretin therapy, as discussed above, may result in potential advantages that may be observed by using this combination as a glucose-lowering strategy when treating people with type 2 diabetes. The main advantages are: (1) the combined reduction of fasting and postprandial glycemia which will lower HbA1c; (2) the lower risk of hypoglycemia which is due to the protection against hypoglycemia with incretin therapy in association with the often observed reduction in insulin dose when using this combination; (3) the lower risk for weight gain, which again is due to the protection against weight gain by incretin therapy in association with reduced weight gain through reduction in the insulin dose; and (4) the potential long-term disease modifying prospect of the combination.

CLINICAL STUDIES OF ADDING GLP-1 RECEPTOR AGONISTS TO INSULIN

Exenatide

The first proper clinical trial exploring the combination of incretin therapy with insulin was a study in 259 patients with type 2 diabetes who were treated with insulin glargine (± metformin and/or pioglitazone) with insufficient glycemic control (HbA1c 7.5%-10.5%; mean 8.4%). Patients were randomized to receive additional therapy with exenatide BID (n = 138) or placebo (n = 123) and the dose of insulin glargine was titrated to achieve a fasting glucose level less than 5.6 mmol/L[55]. After the study period of 30 wk, HbA1c was reduced by 1.7% in the group treated with exenatide BID as an add-on compared to 1.0% by placebo ($P < 0.001$). The daily insulin glargine dose had increased by 20 U (95%CI: 16-24) in the placebo group and by 13 U (95%CI: 9-17) in the exenatide BID-group ($P = 0.030$) (baseline insulin glargine dose was 48 U). Postprandial glucose was reduced in the exenatide BID-treated group (by 2.0 mmol/L, 95%CI: 1.5-2.5 mmol/L) but not changed in the placebo group ($P < 0.001$), whereas changes in fasting glucose did not differ between the two groups. Body weight was reduced (by 1.8 kg) in the exenatide BID group but increased (by 1.0 kg) in the placebo-treated group (baseline 94 kg). Furthermore, the number of hypoglycemic events did

Table 1 Published clinical trials with glucagon-like peptide-1 receptor agonists added to ongoing insulin therapy

		Exenatide BID		Lixisenatide	
Ref		56	58	59	60
Number of patients		259	495	446	311
Duration (wk)		30	24	24	24
HbA1c	Baseline	8.3 ± 0.9	8.4 ± 0.9	7.6 ± 0.5	8.5 ± 0.7
	Change	-1.7 (-1.9, -1.6)	-0.7 ± 0.1	-0.7 ± 0.1	-0.8 ± 0.2
	Baseline comparator	8.5 ± 1.0	8.4 ± 0.8	7.6 ± 0.5	8.5 ± 0.8
	Change comparator	-1.0 (-1.2, -0.9)	-0.4 ± 0.1	-0.4 ± 0.1	+0.1 ± 0.2
FPG (mmol/L)	Baseline	7.9 ± 2.1	8.1 ± 2.8	6.6 ± 1.7	7.8 ± 2.2
	Change	-1.6 (-1.9, -1.3)	-0.6 ± 0.2	-0.3 ± 0.2	-0.4 ± 0.3
	Baseline comparator	8.3 ± 2.3	8.0 ± 2.7	6.7 ± 2.0	7.7 ± 2.3
	Change comparator	-1.5 (-1.8, -1.2)	-0.6 ± 0.3	-0.5 ± 0.2	0.3 ± 0.3
Hypoglycemia	GLP-1RA	1.4[1]	206[2]	28[3]	42[3]; 33[4]
	Comparator	1.2[1]	522[2]	22[3]	24[3]; 28[4]
Body weight	Baseline	95 ± 20	89 ± 21	88 ± 22	66 ± 13
	Change	-1.8 (-2.4, -1.1)	-1.8 ± 0.2	0.3 ± 0.3	-0.4
	Baseline comparator	93 ± 21	88 ± 20	87 ± 21	66 ± 12
	Change comparator	1.0 (0.2, 1.7)	-0.5 ± 0.3	1.2 ± 0.3	+0.1

Insulin glargine was used in all studies. Occurrence of hypoglycemia was reported as number of episodes per patient year[1], number of events[2] or as percentage of patients with at least one hypoglycemic episode[3]. One study also reported percentage of patients not on sulfonylurea who experienced at least one hypoglycemic episode[4]. Variation in baseline is SD, variation in effect is SE. Variation within parenthesis is the 95%CI. FPG: Fasting glucose; GLP-1: Glucagon-like peptide-1; HbA1c: Hemoglobin A1c.

not differ significantly between the groups inspite of the difference in HbA1c (1.4 episodes per patient year in the exenatide BID-treated group *vs* 1.2 episodes per patient year in the placebo group) (Table 1).

In another study, a direct comparison was performed between adding exenatide BID *vs* short-acting prandial insulin lispro to ongoing insulin glargine (+ metformin) in patients who were inadequately controlled on insulin glargine + metformin. The study used an initial 12 wk titration phase with insulin glargine [fasting glucose (FPG) glucose target < 5.6 mmol/L]. Patients who failed to reduce HbA1c below 7% during this titration period (mean 8.3%) were randomized to receive additional exenatide BID (n = 316) or insulin lispro (n = 321). The results showed that after 30 wk, HbA1c had been reduced by 1.1% ± 0.1% in both groups (not significantly different). Fasting glucose was reduced by 0.5 ± 0.2 mmol/L in the exenatide group *vs* 0.2 ± 0.2 mmol/L in the insulin lispor group (P = 0.002) and whereas postprandial glucose was similarly reduced after breakfast and evening meals, it was more pronouncedly reduced by lispro at lunch (when exenatide was not given; P < 0.001). Body weight was reduced in the exenatide BID group (by 2.4 ± 0.2 kg) and increased in the insulin lispro group (by 2.1 ± 0.2 kg). The number of hypoglycemic events was lower in the exenatide group (n = 206) than in the insulin lispro group (n = 522)[56].

Lixisenatide

The GLP-1 receptor agonist lixisenatide has been examined as an add-on to basal insulin in three studies. In the first study, patients treated with basal insulin with inadequate glycemic control (HbA1c 7%-10%, mean 7.6%) were randomized to addition of lixisenatide (n = 328) or placebo (n = 167) without any insulin titration[57]. The used basal insulins in the study were insulin glargine (50%), insulin detemir (47%), neutral protamine Hagedorn (NPH) insulin (7%) or premix insulin (2%) and 80% of the patients were additionally treated with metformin. After the study period of 24 wk, HbA1c was reduced by 0.7% by lixisenatide and by 0.4% by placebo (P < 0.001). Fasting glucose was reduced in both groups but with no significant difference. In contrast, postprandial glucose was more pronouncedly reduced in the lixisenatide group (by 5.5 ± 0.5 mmol/L) than in the placebo group (by 1.7 ± 0.5 mmol/L, P < 0.001). Body weight (from baseline of 88 kg) was reduced by 1.8 kg by lixisenatide and 0.5 kg by placebo (P < 0.001). The daily insulin dose (mean 56 U at baseline) had been reduced by 5 U in the lixisenatide group and by 2 U in the placebo group. Twenty-eight percent of patients in the lixisenatide group reported hypoglycemia *vs* 22% in the placebo group.

In the second study on add-on with lixisenatide to basal insulin, lixisenatide was added to insulin glargine in patients who initially failed to control glycemia with oral agents (HbA1c 7%-10%, mean HbA1c 8.6%)[58]. After an initial titration phase of insulin glargine alone for 12 wk targeting a fasting glucose of 4.4-5.6 mmol/L (mean HbA1c was reduced to 7.6%), patients were randomized to lixisenatide (n = 223) or placebo (n = 223) together with ongoing insulin therapy (+ metformin) for 24 wk. It was found that mean HbA1c was further reduced to 7.0% in the lixisenatide group *vs* to 7.3% in the placebo group (P < 0.001). Fasting glucose was similarly reduced in both groups, whereas postprandial glucose was reduced more in the lixisenatide group (by 3.4 ± 0.5 mmol/L) than in the placebo group (0.1 ± 0.5 mmol/L; P < 0.001). Body weight was increased by 1.2 kg in the placebo group and by 0.3 kg in the lixisenatide group (baseline 86 kg) (P = 0.0012). Confirmed hypoglycemia was reported in 0.80 episodes per patient year in the lixisenatide group *vs* 0.44 in the placebo group.

Finally, the effect of adding lixisenatide to ongoing insulin therapy has also been examined in Asian patients with inadequate glycemic control on basal insulin [with (70%) or without sulfonylurea therapy][59]. Of the patients, 60% were treated with insulin glargine, 27% with insulin detemir and 13% with NPH insulin with a mean daily insulin dose of 25 U. The patients were randomized to addition of lixisenatide (n = 154) or placebo (n = 157) together with ongoing therapy with basal insulin ± sulfonylurea. After the 24 wk study period, HbA1c was reduced by 0.8% in the lixisenatide group *vs* increased by 0.1% in the placebo group (P < 0.001). There was a reduction in fasting glucose in the lixisenatide group compared to the placebo group (P = 0.0187) and postprandial

Table 2 Published clinical trials with dipeptidyl peptidase-4 inhibitors combined with basal ± prandial insulin

		Vildagliptin		Sitagliptin		Alogliptin	Saxagliptin	Linagliptin
Ref		62	63	64	65	67	69	70
Number of patients		296	449	641	124	390	455	1261
Study duration (wk)		24	24	24	24	26	52	24
Comparator		Stable insulin	Stable insulin	Stable insulin	Increasing insulin	Stable insulin	Stable insulin	Stable insulin
HbA1c (%)	Baseline	8.4 ± 1.0	8.8 ± 1.0	8.7 ± 0.9	9.2 ± 1.0	9.3 ± 1.1	8.7 ± 0.9	8.3 ± 0.1
	Change	-0.5 ± 0.1	-0.8 ± 0.1	-0.6 (-0.7, -0.5)	-0.6 (-0.9, -0.3)	-0.7	-0.8 ± 0.1	-0.6 ± 0.1
	Baseline placebo	8.4 ± 1.1	8.8 ± 1.0	8.6 ± 0.9	9.2 ± 1.1	9.3 ± 1.1	8.6 ± 0.9	8.3 ± 0.1
	Change placebo	-0.2 ± 0.1	-0.1 ± 0.1	0 (-0.1, 0.1)	-0.2 (-0.5, 0.3)	-0.1	-0.4 ± 0.1	-0.1 ± 0.1
FPG (mmol/L)	Baseline	9.3 ± 3.1	9.6 ± 2.6	9.8 ± 2.9	9.0 ± 3.3	10.3 ± 3.9	NR	8.2 ± 2.6
	Change	-0.8 ± 0.3	-0.8	-1.0 (-1.4, -0.7)	-1.0 (-2.7, -0.2)	-0.6 ± 0.3		-0.2 ± 0.2
	Baseline placebo	8.7 ± 3.1	9.1 ± 2.5	9.9 ± 3.3	8.4 ± 2.8	10.9 ± 4.3	NR	8.4 ± 2.6
	Change placebo	-0.2 ± 0.4	-0.2	-0.2 (-0.6, 0.2)	-1.3 (-1.8, -0.5)	0.3 ± 0.3		-0.3 ± 0.2
Hypoglycemia		113[1]	8.4[2]	16[2]	7[2]	20[2]	23[2]	23[2]
Hypoglycemia placebo		185[1]	7.2[2]	8[2]	14[2]	40[2]	27[2]	22[2]
Body weight (kg)	Baseline	95 ± 2	78 ± 16	87 ± 19	69 ± 12	87 ± 19	88 ± 18	BMI (31 ± 5)
	Change	1.3 ± 0.3	0.1	-0.1 (-0.2, 0.4)	-0.7 (-1.4, -0.1)	0.6 ± 0.2	0.8	-0.2 ± 0.1
	Baseline placebo	95 ± 2	79 ± 17	87 ± 18	66 ± 10	91 ± 21	86 ± 16	BMI (31 ± 5)
	Change placebo	0.6 ± 0.3	-0.4	-0.1 (-0.3, 0.4)	1.1 (0.2, 1.8)	0.6 ± 0.2	0.5	0.1 ± 0.1

In the studies long and medium acting insulin and premixed insulins were used. Occurrence of hypoglycemia was reported as number of events[1] or as percentage of patients with at least one hypoglycemic episode[2]. Variation in baseline is SD, variation in effect is SE. Variation within parenthesis is the 95%CI. FPG: Fasting glucose; BMI: Body mass index (kg/m^2); HbA1c: Hemoglobin A1c.

glucose was reduced by 8 mmol/L in the lixisenatide group but not changed in the placebo group ($P < 0.001$). Symptomatic hypoglycemia was more frequent with lixisenatide (42.9%) *vs* placebo (23.6%). In contrast, in patients not treated with sulfonylurea, hypoglycemia was similar between groups (32.6% *vs* 28.3%, respectively). Change in body weight was not significantly different between the groups whereas the daily insulin dose was reduced by 1.4 U in lixisenatide group *vs* by 0.1 U in the placebo group ($P = 0.0019$).

Albiglutide

A study compared the effects of the once weekly GLP-1 receptor agonist albiglutide ($n = 285$) *vs* insulin lispro ($n = 281$) to ongoing insulin glargine therapy (+ oral agents, no sulfonylurea) in patients with type 2 diabetes with inadequate glycemic control (mean HbA1c 8.5%)[60]. There was a titration algorithm for insulin glargine to achieve fasting glucose of 4.4-7.2 mmol/L. After the 26 wk study period, HbA1c was similarly reduced by albiglutide (0.8% ± 0.1%) and by insulin lispro (0.7% ± 0.2%). Fasting glucose was reduced in both groups with no significant difference. Body weight (baseline 92 kg) was reduced 0.7 ± 0.2 kg by albiglutide and increased by 0.8 ± 0.2 kg by insulin lispro ($P < 0.001$). Mean insulin glargine dose did not change during the study. Thirty-two percent of patients on albiglutide experienced hypoglycemia *vs* 50% with insulin lispro.

CLINICAL STUDIES OF ADDING DPP-4 INHIBITORS TO INSULIN

Vildagliptin

The first study examining a DPP-4 inhibitor in combination with insulin added vildagliptin (*vs* placebo) to insulin treated patients with insufficient glycemic control (HbA1c 7.5%-11%, mean HbA1c 8.4%; $n = 296$)[61]. Patients were treated with basal and prandial insulin (mean 2.8 injections per day, mean daily insulin dose 82 U). After the 24 wk study period, HbA1c was reduced by 0.5% in the vildagliptin group *vs* 0.2% in the placebo group (baseline 8.4%) ($P = 0.01$). During the course of the study, there were 113 hypoglycemic events in the vildagliptin group compared to 185 in the placebo group and whereas there were 6 episodes of severe hypoglycemia in the placebo group, no severe hypoglycemic episode was seen in the vildagliptin group. The mean daily insulin dose was reduced by 1.9 U in the vildaglitpin *vs* increased by 2.4 U in the placebo group. Change in body weight did not differ between the groups (Table 2).

Another study examined the addition of vildagliptin to ongoing insulin (+ metformin) therapy in 449 patients over 24 wk[62]. The patients were treated with long-acting insulin (22%), intermediate acting insulin (17%) and premixed insulin (60%), with a mean daily insulin dose of 40 U. They had insufficient glycemic control (HbA1c 7.5%-11%; mean HbA1c 8.8%). It was found that HbA1c was reduced by vildagliptin by 0.8% and by placebo by 0.1% ($P < 0.001$). Fasting glucose was reduced in the vildagliptin group but not in the placebo group ($P = 0.050$). Hypoglycemia was reported in 8.4% of patients in the vildagliptin group and by 7.2% in the placebo group. The daily insulin dose was 41 U at baseline and slightly reduced in both groups with no difference. There was no change in body weight in any of the groups.

Sitagliptin

The first study examining the combination of sitagliptin with insulin therapy added the DPP-4 inhibitor *vs* placebo to ongoing insulin (+ metformin) treatment over 24 wk in 641 patients with poorly controlled type 2 diabetes

(HbA1c 7.5%-11%, mean HbA1c 8.6%)[63]. Seventy-four percent of the patients were treated with long-acting or intermediate-acting insulin and 26% were treated with premixed insulin. After the 24 wk study period, HbA1c was reduced by 0.6% by sitagliptin *vs* no change by placebo ($P < 0.001$). Fasting glucose was reduced in the sitagliptin group but not in the placebo group ($P < 0.001$). Similarly, postprandial glucose was reduced in the sitagliptin group (by -1.7 mmol/L, 95%CI: -2.2, -1.2) but not changed in the placebo group (0.3 mmol/L, 95%CI: -0.2-0.7) ($P < 0.001$). Hypoglycemia was observed in 16% of the patients on sitagliptin *vs* 8% of patients on placebo. Insulin dose was reduced by 0.1 U in the sitagliptin and by 1.6 U in the placebo group (baseline 44 U for long acting insulin and 67-74 U with premixed insulin). Body weight was reduced by 0.1 kg in both groups.

Another study compared adding sitagliptin to insulin therapy *vs* increasing the insulin dose in 140 patients on insulin therapy (+ oral agents) who had inadequate glycemic control (baseline HbA1c 7.5%-11%, mean HbA1c 9.2%). Patients were treated with insulin glargine alone (48%), insulin glargine together with rapid acting insulin (23%) or NPH insulin in combination with regular insulin (29%); mean daily insulin dose was 37 U. It was found that over the 24 wk study period, sitagliptin (mean insulin dose reduced by 2 U) reduced HbA1c by 0.6%, whereas increasing the insulin dose (by 10 U) reduced HbA1c by 0.2% ($P < 0.005$)[64]. Fasting glucose was reduced by approximately 1 mmol/L in both groups with no significant difference. Hypoglycemia occurred in 7 events per patient year in the sitagliptin group *vs* 14.3 events per patient year in the insulin group. Body weight was reduced by 0.7 kg in the sitagliptin group *vs* increased by 1.1 kg in the insulin group ($P < 0.05$).

A third study examined the add-on of sitagliptin ($n = 236$) *vs* placebo ($n = 232$) to patients who were treated with insulin (long-acting, intermediate-acting or premixed insulin) in combination with metformin over 6 mo. It was found that with the addition of sitagliptin, HbA1c was reduced by 0.8% (baseline 8.5%) *vs* no change in HbA1c after addition of placebo ($P < 0.001$). Relative to the placebo group, fasting glucose was reduced by 1.0 mmol/L and postprandial glucose by 2.0 mmol/L. Hypoglycemia was observed in 18% of patients in the sitagliptin group *vs* 8% in the placebo group[65].

Alogliptin

Alogliptin (two doses) or placebo was added to ongoing insulin therapy alone (40%) or with metformin in 390 patients with inadequate glycemic control (HbA1c $\geqslant$ 8.0%; baseline HbA1c 9.3%)[66]. The insulin treatment that was used was premixed insulin or insulin combinations (64%), as well as long-acting basal insulin alone (34%) or short-acting insulin alone (2%); mean daily insulin dose was 57 U. During the course of the 26 wk study, daily insulin dose was kept constant. Alogliptin reduced HbA1c by 0.6% (12.5 mg daily; $n = 131$) and 0.7% (25 mg daily; $n = 129$) *vs* a reduction by 0.1% in the placebo group ($n = 130$) ($P < 0.001$). Fasting glucose was reduced by aloglitin in the 25 mg group (by -0.6 ± 0.3 mmol/L *vs* the placebo group (0.3 ± 0.3 mmol/L; $P = 0.030$) but not changed in the 12.5 mg group. The number of patients reporting hypoglycemia was lower in the two alogliptin groups (21% and 20%, respectively) than in the placebo group (40%; $P < 0.001$). There was no difference in hypoglycemia events (24%-27% of patients reported hypoglycemic episodes in the three groups). Body weight increased by 0.6 kg (baseline 88 kg) in all groups.

Saxagliptin

Saxagliptin or placebo was added to ongoing insulin therapy (basal insulin or premixed insulin ± metformin) in 455 patients with inadequate glycemic control (HbA1c 7.5-11). During the course of the 24 wk study, daily insulin dose was kept constant[67]. Placebo-adjusted reduction in HbA1c by saxagliptin was 0.4% ($P < 0.001$). There was no difference in hypoglycemia events (18% with saxagliptin, 20% with placebo). Body weight was increased by 0.4 kg in the saxagliptin group and by 0.2 kg in the placebo group. An extension phase of this study showed sustained effects over 52 wk[68].

Linagliptin

Linagliptin or placebo was added to ongoing basal insulin therapy (± metformin and/or pioglitazone) in 1261 patients with inadequate glycemic control (HbA1c 7-10). During the study, daily insulin dose was kept constant during the first 24 wk but could thereafter be titrated according to fasting glucose[69]. After 24 wk, HbA1c was reduced by 0.6% (baseline 8.3%) by linagliptin and by 0.1% by placebo ($P < 0.001$). Placebo-adjusted reduction in fasting glucose with linagliptin was 0.6 mmol/L (95%CI: -0.9-0.4). During the following 28 wk, insulin dose was increased by 2.6 U in the linagliptin group and by 4.2 U in the placebo group but with no further change in HbA1c. There was no difference in hypoglycemia events (23% with linagliptin, 22% with placebo after 24 wk). Body weight was reduced by 0.3 kg in the linagliptin group and by 0.04 kg in the placebo group.

COMPARING CONTROLLED TRIALS COMBINING ADDING INCRETIN THERAPY TO INSULIN

As outlined above, the reduction in HbA1c, fasting and postprandial glucose, the lower risk of hypoglycemia, the prevention of weight gain and the potential disease modification are the main advantages of combining incretin therapy with insulin. Except for any direct evidence of a disease modifying effect of the combination, the controlled trials summarized above include information on these aspects and therefore it is of interest to compare their results in this regard (Tables 1 and 2).

HbA1c

The mean reduction in HbA1c in the controlled clinical studies adding incretin therapy to stable dose for 6 mo

was -0.8% ± 0.1% compared to -0.3% ± 0.1% when placebo was added ($P < 0.001$; Tables 1 and 2). There does not seem to be a difference between the two different strategies of incretin therapy since the placebo-adjusted reduction in HbA1c was -0.6% ± 0.2% for GLP-1 receptor agonists ($n = 4$ studies) *vs* -0.5% ± 0.1% for DPP-4 inhibitors ($n = 6$ studies).

Fasting glucose

Fasting glucose is also reduced by adding incretin therapy to stable dose of insulin. It was found to be reduced by -0.7 ± 0.1 mmol/L by the incretin therapy *vs* by -0.3 ± 0.1 mmol/L in the placebo groups ($P = 0.027$; Tables 1 and 2). There does not seem to be a difference between the two different strategies of incretin therapy since fasting glucose was reduced by 0.2 ± 0.2 mmol/L by GLP-1 receptor agonists ($n = 4$ studies) *vs* by -0.6 ± 0.2 mmol/L by DPP-4 inhibitors ($n = 5$ studies).

Postprandial glucose

A few studies also examined postprandial glucose after adding incretin therapy to a stable dose of insulin. They showed that postprandial glucose was markedly reduced when adding GLP-1 receptor agonists exenatide BID[55] and lixisenatide[58], whereas after adding the DPP-4 inhibitor sitagliptin, postprandial glucose was more modestly reduced[63].

Hypoglycemia

In the studies where incretin therapy has been added to insulin compared to ongoing insulin, the occurrence of hypoglycemia was not different between the incretin treatment and placebo in most studies (Tables 1 and 2). Since in most of these studies HbA1c is lower after addition of incretin therapy compared to placebo, an increased risk of hypoglycemia would be expected after incretin therapy. Since the opposite was the case, a conclusion is that incretin therapy will reduce the risk of hypoglycemia. This conclusion is also evident in the studies in which incretin therapy as an add-on to basal insulin was compared with the active comparator of either adding short-acting insulin[56,60] or increasing the insulin dose[64]. A reason for the low risk of hypoglycemia when adding incretin therapy to insulin therapy could be the reduced dose of insulin which often accompanies the combination. It may, however, also be caused be a sustainment of the glucagon counterregulation to hypoglycemia, as was recently demonstrated for the DPP-4 inhibitor vildagliptin when added to insulin; the sustained glucagon counterregulation assures a sufficient hepatic glucose response to prevent hypoglycemia[50].

Weight gain

Body weight was significantly reduced by -0.9 ± 0.5 kg by adding GLP-1 receptor agonists to ongoing insulin therapy compared to 0.4 ± 0.4 kg in the placebo groups, corresponding to a placebo-adjusted reduction by -1.4 ± 0.5 kg (Table 1). In contrast, DPP-4 inhibitors are weight neutral when added to insulin with a placebo-adjusted change in body weight of -0.2 ± 0.1 kg (Table 2).

OTHER STUDIES COMBINING INCRETIN THERAPY WITH INSULIN

Adding insulin to a GLP-1 receptor agonist

One study has examined the addition of basal insulin to patients who are treated with a GLP-1 receptor agonist with insufficient glycemic control. The study initially examined addition of liraglutide to patients failing glycemic control on metformin (± sulfonylurea; sulfonylurea was removed at start of study) ($n = 988$)[70]. After 12 wk, patients who were still uncontrolled (HbA1c > 7%) were randomized to continue metformin plus liraglutide or addition of insulin detemir to titrate fasting glucose to 4-6 mmol/L. After another 26 wk, HbA1c had been reduced by 0.5% by the combination of insulin detemir plus liraglutide, whereas those on liraglutide alone (all with metformin) had no further change in HbA1c ($P < 0.001$). FPG decreased more in the liraglutide + insulin group than in the liraglutide control group ($P < 0.001$). Hypoglycemia rates were 9.2% in the group given insulin detemir and liraglutide *vs* 1.3% with liraglutide alone. Body weight (baseline 96 kg) decreased by 3.5 kg by liraglutide during the initial period and then by 0.16 kg with insulin detemir and liraglutide *vs* by 0.95 kg with liraglutide without insulin detemir ($P = 0.03$).

Initial combination of incretin therapy with insulin

Liraglutide has been examined in a fixed ratio combination with insulin degludec in a randomized study in subjects with type 2 diabetes[71]. It was a large trial in which patients treated with metformin ± pioglitazone and inadequate glycemic control (baseline HbA1c 8.3%) were randomized to the addition of insulin degludec ($n = 414$), liraglutide ($n = 415$) or the combination of insulin degludec and liraglutide ($n = 834$). After 26 wk of treatment, HbA1c had been reduced by 1.4% with insulin degludec alone, 1.3% with liraglutide alone and 1.9% with insulin degludec in combination with liraglutide. Body weight had increased by 2.2 kg with insulin degludec alone, was reduced by 2.4 kg by liraglutide and was neutral with the combination. Cumulative episodes of hypoglycemia were 1.3 per patient in the insulin degludec group and reduced to 0.9 per patient in the combination group (0.1 in the liraglutide alone group).

Another study randomized 217 patients who had insufficient glycemic control on metformin ± sulfonylurea to receiving sitagliptin plus sulfonylurea or sitagliptin plus insulin detemir (all on metformin). After the 26 wk study period, sitagliptin had reduced HbA1c by 0.9% (mean baseline HbA1c 8.5%), whereas sitagliptin plus insulin detemir had decreased HbA1c by 1.4%. Hypoglycemia was reported in 1.3% of patients in the insulin detemir plus sitagliptin group and 1.7% in the sitagliptin alone group. Body weight decreased in both arms with a mean decrease of -1.7 kg in the sitagliptin control group *vs* -0.8

kg with sitagliptin plus insulin detemir group[72].

Uncontrolled studies combining incretin therapy with insulin

There are also a few uncontrolled studies of combining insulin therapy and incretin therapy in patients with type 2 diabetes which arrive at similar conclusions as the previously summarized controlled trials. One retrospective report showed that addition of exenatide BID to 188 insulin-treated patients resulted in a reduction in HbA1c by 0.66% (baseline 8.1%) after 6 mo with a persistent effect throughout two years; mean insulin dose could be reduced by 15% and only 4% of patients experienced hypoglycemia[73]. Furthermore, a study in obese patients with type 2 diabetes added exenatide BID (n = 21) or liraglutide (n = 40) to ongoing insulin therapy and showed a reduction in HbA1c in these patients by 1.0% (baseline 8.9%) after 7 mo. At the same time, the daily insulin dose was reduced from 91 U to 52 U and only a few hypoglycemia episodes were reported[74]. Moreover, a study in severely insulin resistant obese subjects treated with insulin U-500 (mean daily dose 192 U) received liraglutide for twelve weeks which reduced HbA1c by 1.4% (mean baseline HbA1c 8.5%) and at the same time the insulin dose was reduced by 28%. There were no reports of hypoglycemia and body weight was reduced by 5 kg (baseline body weight 136 kg)[75].

SAFETY OF THE COMBINATION OF INSULIN AND INCRETIN THERAPY

Incretin therapy has been shown to be safe with high tolerability and the only consistent adverse event is nausea and vomiting during the initial treatment period with GLP-1 receptor agonists[17-20,76]. Local injection site reactions (nodules and/or erythema) sometimes occur in association with treatment with GLP-1 receptor agonists, although such reactions are rare and commonly transient. Antibodies may be formed against GLP-1 receptor agonists; more commonly with exendin-4-based agonists (exenatide, lixisenatide) than after GLP-1-based agonists. In contrast, adverse events are rare during treatment with DPP-4 inhibitors, as evident from pooled analysis of clinical trials[77,78]. Recently, there has been a discussion about whether there is an increased risk of acute pancreatitis in incretin therapy. However, pooled or meta-analysis analyses have not demonstrated any increased risk when compared to placebo or other comparators[76-79]. Nevertheless, it is important to follow patients on GLP-1 receptor agonists in this regard and in patients with a history of acute pancreatitis, incretin therapy should not be given. Rodent data also suggest that GLP-1 receptor agonists may be associated with medullary thyroid carcinoma[80]. This has not, however, been observed in other animal species or humans, possibly because C-cells in humans have a lower expression of GLP-1 receptors than rodent C-cells[81].

Incretin therapy has also been discussed in relationship to cardiovascular safety and meta-analyses have shown that there is no detrimental effect of GLP-1 receptor agonists[82] or DPP-4 inhibitors[83]. Furthermore, several cardiovascular safety trials with incretin therapy are at present ongoing and two such recently published studies showed no increased risk for cardiovascular disease with saxagliptin[84] or alogliptin[85].

Also, insulin therapy is safe with the only concern being the increased risk of hypoglycemia and weight gain, expected adverse events through the glucose-lowering actions of the therapy. By combining incretin therapy and insulin, there is no additional concern for safety or tolerability, as evident from the studies reported in this review[55-69]. Hence, the number of adverse events is not higher in the incretin therapy + insulin treatment groups than in placebo groups in placebo-controlled clinical trials on GLP-1 receptor agonists or DPP-4 inhibitors as an add-on to insulin therapy, except the nausea and vomiting for the GLP-1 receptor agonists. This also includes recently discussed potential adverse events such as acute pancreatitis.

Some practical aspects need to be taken into account for incretin therapy. This includes the dose reduction of sitagliptin, vildagliptin, saxagliptin and alogliptin in patients with renal impairment due to their renal excretion. Furthermore, exenatide and liraglutide should be cautiously used in patients with renal impairment due to insufficient experience in this patient group. Furthermore, in patients with hepatic impairment, vildagliptin is not recommended. As for all new treatment combinations, however, the combination of incretin therapy with insulin also needs careful follow-up for examining potential adverse events which have not yet been observed.

SUMMARY AND CLINICAL POSITIONING OF INCRETIN PLUS INSULIN COMBINATION

The combination of insulin therapy with incretin therapy is attractive due to experience that this combination improves glycemia with a low risk of increasing risk for hypoglycemia and low risk of weight gain. The combination is therefore of particular value in patients on insulin therapy in whom HbA1c is not sufficiently reduced. In some patients, insufficient improvement of glycemia may be caused by clinical inertia with reluctance to increase the insulin dose due to fear of hypoglycemia or weight gain. Addition of incretin therapy with its lower risk of hypoglycemia and low risk of weight gain may therefore prevent the clinical inertia in these patients.

Incretin addition is also of value in patients who have insufficient reduction in HbA1c by intensified basal insulin therapy due to persistent high postprandial glycemia or frequent hypoglycemia. Incretin therapy offers advantages over addition of prandial insulin in these patients. Of particular importance is the prevention of hypoglycemia, since hypoglycemia is associated with both short-

term and long-term negative impact, not least on cardiovascular outcomes. The combination of incretin therapy with insulin may therefore provide advantages both in the short-term and by reducing long-term complications to the disease.

A main indication for the combination of incretin therapy and insulin is thus in patients who are treated with basal insulin (± metformin) in whom there is insufficient glycemic control and/or an unacceptable high rate of hypoglycemia and/or unacceptable weight gain. In patients with HbA1c levels which are not very high (< 7.5%), it is advisable to reduce the basal insulin dose when starting incretin therapy. The combination of incretin therapy with insulin is also an important treatment strategy in patients who are treated with metformin and incretin therapy in combination and in whom the glycemic control is insufficient, *i.e.*, to add basal insulin therapy to incretin therapy (+ metformin). The combination with incretin therapy and insulin may thus be introduced in either way, starting with incretin therapy or starting with insulin. It is also a possibility to start immediately with initial combination with incretin therapy and insulin in patients who are treated with metformin and who are in insufficient metabolic control. Such an early introduction of the combination may be a solution to the unmet need to start aggressive therapy early on during the disease development to achieve long-term control. Further studies are required to examine the long-term effects of this initial combination. One important set of trials would be studies comparing this treatment strategy with other three-drug combinations. This would be of interest to further analyze the potential for the combination of incretin plus insulin therapy (+ metformin). What would also be of value would be to compare different incretin therapies (different GLP-1 receptor agonists and different DPP-4 inhibitors) to elucidate potential differences in effects of the different compounds when combined with insulin. More mechanistic studies would also be of value, for example to examine the relationship between insulin therapy and incretin hormones for the regulation of hepatic glucose output, glucose utilization and islet function and, furthermore, to study impact of the combination therapy on gastric emptying and satiety. Moreover, it would also be of great value to analyze the cardiovascular outcome of this three-drug combination. This would be possible in sub-group analysis on the cardiovascular outcome trials of incretin treatment in which patients on insulin therapy have also been enrolled. Finally, studies directly aiming at examining the potential disease modifying effect of the combination of incretin therapy and insulin are important; these studies need to have a long duration and include mechanistic studies on islet function.

The combination of incretin therapy with insulin (± metformin) is thus a promising glucose-lowering strategy in type 2 diabetes, allowing a more intensified treatment at an earlier stage of the disease with a lower risk for hypoglycemia and weight gain when compared to other intensifying therapies.

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MINIREVIEWS

Hepatitis C virus infection and insulin resistance

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Abstract

Approximately 170 million people worldwide are chronically infected with hepatitis C virus (HCV). Chronic HCV infection is the leading cause for the development of liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and is the primary cause for liver transplantation in the western world. Insulin resistance is one of the pathological features in patients with HCV infection and often leads to development of type Ⅱ diabetes. Insulin resistance plays an important role in the development of various complications associated with HCV infection. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, HCC and resistance to anti-viral treatment. Thus, HCV associated insulin resistance is a therapeutic target at any stage of HCV infection. HCV modulates normal cellular gene expression and interferes with the insulin signaling pathway. Various mechanisms have been proposed in regard to HCV mediated insulin resistance, involving up regulation of inflammatory cytokines, like tumor necrosis factor-α, phosphorylation of insulin-receptor substrate-1, Akt, up-regulation of gluconeogenic genes like glucose 6 phosphatase, phosphoenolpyruvate carboxykinase 2, and accumulation of lipid droplets. In this review, we summarize the available information on how HCV infection interferes with insulin signaling pathways resulting in insulin resistance.

Key words: Hepatitis C virus; Insulin resistance; Insulin receptor substrate 1; Protein kinase B; mammalian target of rapamycin/S6K1; Suppressor of cytokine signaling 3; Glucose transporter-4; Lipid metabolism; Anti-viral therapy

Core tip: Insulin resistance is one of the pathological features in patients with hepatitis C virus (HCV) infection and often leads to development of type Ⅱ diabetes. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, hepatocellular carcinoma and resistance to anti-viral treatment. In this review, we summarize the available information on how HCV infection interferes with insulin signaling pathways.

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INTRODUCTION

Hepatitis C virus (HCV) contains a positive sense single stranded RNA genome and belongs to the family Flaviviridae and genus Hepacivirus[1]. HCV genome, 9.6 kb in length, is composed of a 5' non-translated region (NTR), a long open reading frame (ORF) encoding a polyprotein and a 3' NTR. The ORF encodes a polyprotein of about 3000 amino acids that is translated *via* an internal ribosome entry site at the 5' NTR. The polyprotein is then cleaved by both cellular and viral proteases into at least

10 different proteins[1]. These include three structural proteins namely, core and two envelope glycoproteins (E1 and E2). In addition, a protein called F or ARFP can be produced from a frame-shift of the core protein[2]. An ion channel protein p7 is formed by cleavage of E2[3]. Non structural proteins of HCV include NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The primary host cell for HCV is hepatocytes but replication may also occur in other cell types, such as peripheral blood mononuclear cells, as well as in B and T cell lines[4,5]. HCV is a major cause of acute and chronic liver disease worldwide. More than 170 million people are currently infected with HCV[6]. Currently HCV vaccine is not available. Acute infection is usually asymptomatic, making early diagnosis difficult. Approximately 70% of acutely infected individuals fail to clear the virus and become chronically infected[7]. Chronic HCV infection is the leading cause for the development of liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and is the primary cause for liver transplantation in the western world. The sustained antiviral response rate in treatment of chronic HCV infection with interferon (IFN)-α with ribavirin is limited (about 30%-40%)[8,9]. Boceprevir and telaprevir protease inhibitors, have been shown to exhibit significantly higher rates of sustained virologic response (SVR) against HCV genotype 1 (about 65%-75%) as compared with peginterferon-ribavirin alone[10,11]. However, use of these antiviral agents display higher incidence of adverse events, such as rash, gastrointestinal disorders, and anemia.

Insulin resistance plays an important role in the development of various complications associated with HCV infection. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, HCC and resistance to anti-viral treatment[12]. Thus, HCV associated insulin resistance is a therapeutic target at any stage of HCV infection. HCV modulates normal cellular gene expression and interferes with the insulin signaling pathway. The aim of this review is to summarize the currently available information on how chronic HCV infection interferes with insulin signaling pathways resulting in insulin resistance.

GLUCOSE UPTAKE AND INSULIN RESISTANCE

Glucose is a key metabolite essential for the production of energy (mostly ATP) which is required by cells. There are several mechanisms underlying increased glucose production. These include production of free glucose by increased glycogenolysis in the liver, increased gluconeogenesis, activation of forkhead box transcription factor (FoxO1) and improper insulin-glucagon hormonal balance, which stimulates increased glucose production[13]. Several factors contribute to elevated gluconeogenesis in diabetes, namely (1) increased supply of glucogenic precursors to the liver (glycerol, amino acids, free fatty acids), (2) increased lipid content, (3) increased cytokines and adipokines, and (4) decreased insulin receptor (IR) signaling in hepatocytes[13]. Glucose uptake into cells is regulated by the action of specific hormones, namely insulin and glucagon. Insulin is a peptide hormone secreted by the β-cells of the pancreatic islets of langerhans and maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects[14]. The ability of insulin to stimulate glucose uptake into tissues is central to the maintenance of whole-body glucose homeostasis[15]. Type Ⅱ diabetes mellitus (T2DM), occurs when the production of insulin is not sufficient to overcome a difficulty the body has in properly using insulin. This difficulty is called insulin resistance, resulting in increased glucose levels. Both forms of diabetes can pose an increased risk of major lifelong complications. In the case of insulin resistance, this includes a fivefold increased risk of coronary vascular disease, diabetic retinopathy and neuropathy[16-19]. Fatty liver is relatively common in overweight and obese persons with T2DM and is an aspect of body composition related to severity of insulin resistance, dyslipidemia, and inflammatory markers[20].

Glucose transporter-4 (GLUT-4) was shown to be the major isoform responsible for enhanced glucose uptake into muscle and adipose tissues following the secretion of insulin into the bloodstream[21,22]. The process of glucose uptake by cells requires a series of events to take place in a timely manner. It involves the binding of insulin to the IR resulting in subsequent phosphorylation and activation of IR substrate 1 and 2 (IRS-1/IRS-2), central molecules of the insulin signaling cascade[23,24]. This in turn activates protein kinase B (AKT) by phosphorylation of Ser^{473} and Thr^{308} residues. Activated AKT causes the translocation of GLUT-4 from intracellular compartments to the cell surface where it is required for glucose uptake[25]. Any change in the signaling is likely to induce insulin resistance which is associated with a number of pathophysiological changes including glucose intolerance, obesity, dyslipidemia and hypertension. Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone insulin. The body produces insulin, but the cells in the body become resistant to insulin and are unable to use it as effectively, resulting in an attenuated biological response, leading to hyperglycemia[26]. Accumulation of ectopic lipid metabolites, activation of the unfolded protein response pathway, and innate immune pathways have all been implicated in the pathogenesis of insulin resistance[27]. During the course of insulin resistance several inflammatory cytokines and lipid metabolites, like free fatty acids, interrupt with the normal insulin signaling and promote T2DM.

CHRONIC HCV INFECTION AND INSULIN RESISTANCE

Epidemiological studies suggest that patients with chron-

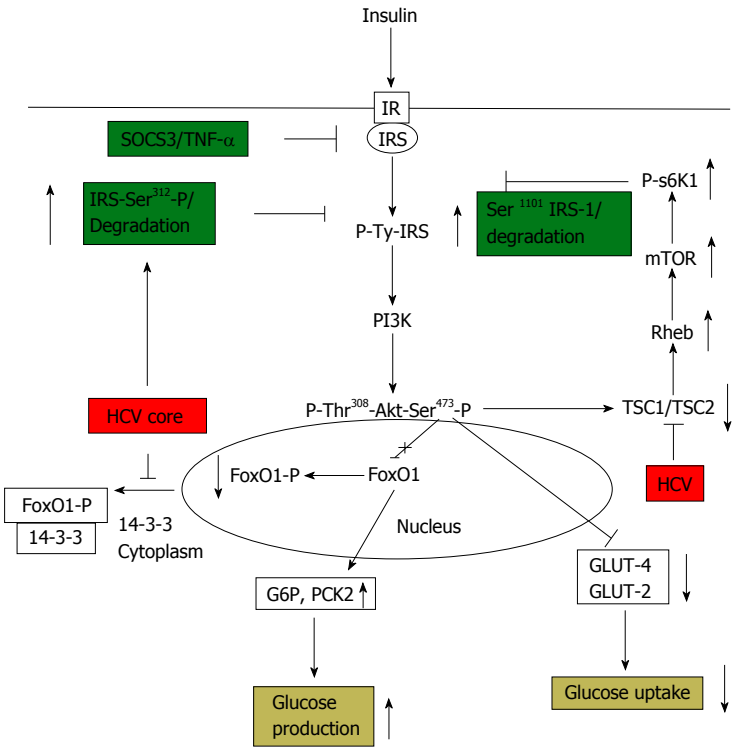

Figure 1 Schematic showing the interference of Hepatitis C virus in the insulin signaling pathway. Hepatitis C virus (HCV) core protein is known to up regulate Ser^{312} phosphorylation of insulin receptor substrate (IRS)-1 leading to degradation of IRS-1, the key molecule involved in propagation of insulin signal downstream from the insulin receptor (IR). HCV infection is also known to down regulate TSC1/TSC2 complex, resulting in subsequent upregulation of mTOR/S6K1 which leads to Ser^{1101} phosphorylation of IRS-1 and its subsequent degradation. A role of HCV mediated upregulation of SOCS3 and tumor necrosis factor-α (TNF-α) has also been proposed which leads to degradation and blocking of IRS-1 function. HCV also upregulates glucose 6 phosphatase (G6P), phosphoenolpyruvate carboxykinase 2 (PCK2) leading to increased glucose production, and down regulates glucose transporter (GLUT)-4, GLUT-2, leading to decreased glucose uptake by hepatocytes. Overall, these alterations lead to insulin resistance. mTOR: Mammalian target of rapamycin.

ic HCV infection have a significantly increased prevalence of T2DM as compared to hepatitis B virus infected patients[28-30]. Both insulin resistance and diabetes can adversely affect the course of chronic hepatitis C (CHC), leading to enhanced steatohepatitis and liver fibrosis[30-32]. Insulin resistance, associated with type 2 diabetes, can promote fatty liver, and excessive hepatic accumulation of fat may promote insulin resistance and therefore contribute to the pathogenesis of the metabolic syndrome[33]. Insulin resistance is a critical component of type 2 diabetes mellitus pathogenesis. Several mechanisms are likely to be involved in the pathogenesis of HCV-related insulin resistance[34]. Several cellular lesions have been associated with insulin resistance, but the precise mechanism by which HCV induces insulin resistance remains elusive with numerous viewpoints and opinions[30].

Impairment of IRS-1 and IRS-2 expression has been observed in the liver of patients with chronic HCV infection, as well as in HCV core transgenic mice, and from *in vitro* cell culture system[35-38]. HCV mediates dysfunction of the insulin signaling pathways *via* several distinct mechanisms, such as upregulating the expression of suppressors of cytokine signaling 3 expression[35], down regulation of peroxisome proliferator-activated receptors gamma (PPARγ)[36], activation of mammalian target of rapamycin (mTOR)/S6K1 pathway[38], and increased tumor necrosis factor-α (TNF-α) secretion[39].

MODULATION OF IR SUBSTRATE BY HCV

HCV modulates insulin signaling and IRS-1 *via* multiple mechanisms which have been presented in Figure 1. Ser/Thr phosphorylation of IRS-1 inhibits its association with the IR, which in turn inhibits tyrosine phosphorylation of IRS-1, required for its activation, and promotes degradation. Upregulation of serine phosphorylation of IRS-1 is a key negative feedback mechanism under physiological conditions to prevent the action of insulin. In an insulin-resistant state, an imbalance occurs between positive IRS-1 Tyr-phosphorylation and negative Ser-phosphorylation of IRS-1[40]. HCV core protein expression in hepatocytes upregulates Ser^{312} phosphorylation status of IRS-1 and modulates downstream Akt activity by inhibiting Thr^{308} phosphorylation[37]. Ser^{312} and Ser^{1101} phosphorylation of IRS-1 inhibits its association with the IR and stimulates degradation. HCV core protein induces insulin resistance by increasing Ser^{312} and Ser^{1101} phosphorylation, marking its for degradation *via* the activated mTOR/S6K1 pathway[38], and subsequently blocking Tyr-phosphorylation of IRS-1 and Thr^{308} phosphorylation of Akt for the inhibition of glucose uptake. Activation of mTOR signaling also plays a key role in modulating IRS-1 activity. HCV genotype 2a infection significantly down-regulates the expression of TSC1/TSC2, which in turn results in activation of downstream mTOR and S6K1[38]. Phosphorylation of IRS-1 at Ser^{1101} *via* the mTOR-S6K1 pathway may release IRS-1 from intracellular complexes, thereby enabling its degradation[41]. HCV significantly increases Ser^{1101} phosphorylation of IRS-1, which enables its degradation[38].

A decrease in expression of IRS-1 and IRS-2, in patients with HCV infection has also been reported[35]. Down-regulation of IRS-1 and IRS-2 was also seen in HCV core-transgenic mice livers and HCV core-transfected human hepatoma cells[35]. HCV core up-regulated suppressor of cytokine signaling 3 (SOCS3) and caused ubiquitination of IRS-1 and IRS-2. HCV core-induced

down-regulation of IRS-1 and IRS-2 was not seen in *SOCS3(-/-)* mouse embryonic fibroblast cells, indicating the important role played by SOCS3 in mediating down regulation of IRS-1[35]. There have been reports that HCV genotypes might play an important role in deciding the pathway by which it impairs insulin signaling. It has been shown that the core protein of HCV genotype 3a promoted IRS-1 degradation through the downregulation of PPARγ and by upregulating the SOCS7, the core protein of genotype 1b activated the mTOR[36].

TNF-α, released in an excess may promote phosphorylation of serine residues of IRS-1 eventually leading to the downregulation of downstream insulin signaling molecule Akt. HCV core protein increases the expression level of TNF-α and promotes insulin resistance[42].

IMPAIRED LIPID AND GLUCOSE METABOLISM BY HCV

Insulin resistance is strongly influenced by abnormalities in lipid metabolism. Any dysfunction of the lipid metabolism triggers lipotoxicity through the production of free fatty acids thereby promoting insulin resistance[43]. HCV core protein down-regulates microsomal triglyceride transfer protein, an enzyme that mediates lipid translocation to the endoplasmic reticulum membrane and decreases the assembly of very low density lipoproteins[44]. It has been observed that HCV promotes fatty acid synthesis by the upregulation of lipogenic gene sterol regulatory element binding protein 1c which promotes the transcriptional activation of other lipogenic genes like acetyl CoA carboxylase, ATP citrate lyase, hydroxymethylglutaryl CoA reductase[45].

HCV infection promotes the expression of gluconeogenic genes namely, glucose 6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase 2 (PCK2) resulting in increased glucose production and enhanced insulin resistance[46,38]. HCV also down regulates the expression of GLUT4, which is necessary for uptake of glucose. This results in a decreased glucose uptake and increased plasma glucose, leading to development of insulin resistance[38].

A schematic showing how HCV interferes with insulin signaling pathway, leading to insulin resistance is presented in (Figure 1). HCV modulates functioning of IRS-1 *via* multiple mechanisms, including up regulation of Ser^{312} or Ser^{1101} phosphorylation which leads to degradation of IRS-1. HCV also upregulates SOCS3 and down regulates TSC1/TSC2 leading to blocking of insulin signaling. HCV infection leads to increased gluconeogenesis *via* up regulation of G6P and PCK2. GLUT-4, and GLUT-2 expression is also down regulated by HCV leading to decreased glucose uptake. Overall, all these alterations by HCV leads to development of insulin resistance.

INSULIN RESISTANCE AND LIVER DISEASE PROGRESSION

The metabolic syndrome is a constellation of problems that includes insulin resistance, obesity, hypertension, and hyperlipidemia[47]. Increasingly, components of the metabolic syndrome are being linked to various forms of cancer, including the risk of developing HCC. IR is induced by HCV-4 irrespective of severity of liver disease. IR starts early in infection and facilitates progression of hepatic fibrosis and HCC development[47]. HCC patients showed higher IR frequency, and moderate to high viral load associated with high HOMA-IR in CHC and HCC[47]. Insulin resistance associates with a higher risk of HCC in cirrhotic HIV/HCV-co-infected patients also[48]. There are many causes of HCC, and nonalcoholic fatty liver disease (NASH) is emerging as a leading risk factor owing to the epidemic of obesity and T2DM. The mechanisms leading to HCC in obesity and T2DM likely involve interactions between several signaling pathways, many of which are modulated by HCV infection, and also include oxidative stress, inflammation, oncogenes, adiponectins, and insulin resistance associated with visceral adiposity and diabetes[49].

Insulin resistance and subsequent hyperinsulinemia are highly associated with fatty liver disease and is an important risk factor for the progression of fibrosis in CHC[50,51]. From metabolic aspect, HCV infection resembles NASH in numerous features, such as the presence of steatosis, serum dyslipidemia, and oxidative stress in the liver[52]. On the other hand, there are noticeable differences between hepatitis C and NASH, in the fact that HCV modulates cellular gene expression and intracellular signal transduction pathways, while such details have not been noted for NASH. HCV core protein expression leads to the development of progressive hepatic steatosis and HCC in transgenic mice[53]. Hepatic steatosis is known to occur at a high rate (40%-86%) in chronic HCV patients, and a close relationship between steatosis and intrahepatic core protein expression has been noted[54]. Insulin resistance is a prominent mechanism linking steatosis and fibrogenesis although this link is complex and not properly understood.

CLINICAL IMPLICATIONS OF HCV-MEDIATED INSULIN RESISTANCE

Several epidemiological, clinical and experimental data show that HCV plays a direct role in perturbing glucose metabolism, leading to both insulin resistance and diabetes[28-30]. Curing HCV results in the amelioration of insulin resistance and decreased incidence of diabetes after the end of therapy[55,56]. In the only trial that used the antidiabetic metformin[57], only a marginal, nonsignificant increase of the SVR rate was observed, despite an increased virological response after 4 wk of triple therapy. The data reported in a study using different schedules containing the antiglycaemic PPAR-γ agonist pioglitazone[58] are discouraging. Overall, the administration of insulin sensitizers together with the standard of care has not only failed to improve the virological response to therapy, but has also fallen short of providing much use-

ful insight into the mechanisms linking reduced response to insulin resistance[59]. Early sulfonylureas although useful in lowering blood glucose level, were associated with significant off-target effects, and the biguanide phenformin was discontinued due to adverse events[60]. Although metformin is in the same drug class, it has a better safety profile and is now recommended as first-line treatment of diabetes during HCV infection.

THERAPEUTIC APPROACHES AND FUTURE GOALS

Treatment for HCV induced insulin resistance is highly linked with anti-viral treatment. Treatment of chronic HCV infection has 2 goals. The first is to achieve SVR (*i.e.*, sustained eradication of HCV, which is defined as the persistent absence of HCV RNA in serum 6 mo or more after completing antiviral treatment). The second goal is to prevent progression to cirrhosis, HCC, and decompensated liver disease requiring liver transplantation. The treatment of HCV has evolved over the years. Current treatment options include combination therapy consisting of ribavirin and pegylated IFN. Protease inhibitors are emerging as a third feature of combination therapy. The sustained antiviral response rate in treatment of chronic HCV infection with IFN-α and ribavirin is limited (about 30%-40%)[8,9]. Boceprevir and telaprevir protease inhibitors have been shown to exhibit significantly higher rates of SVR against HCV genotype 1 (65%-75%) as compared with peginterferon-ribavirin alone[10,11]. More recently, sofosbuvir has also been used for treatment along with ribavirin, with significant increased SVR[61]. However, use of these antiviral agents display higher incidence of adverse events, such as rash, gastrointestinal disorders, and anemia. Thus, development of therapies with less side effects is desirable.

The prevalence of HCV antibodies in the type 2 diabetic population ranges between 1.78% and 12.1%[62]. Several cross-sectional studies have found a higher prevalence of HCV antibodies in type 2 diabetic patients than expected in the general population[62,63]. Early phase and total insulin secretion are determined using oral glucose tolerance testing (OGTT), Insulin sensitivity was measured directly by steady-state plasma glucose concentration during insulin suppression test. Fasting plasma glucose $\geqslant$ 126 mg/dL or 2-h plasma glucose > 200 mg/dL during OGTT are generally used as criteria for diagnosis of diabetes[64]. Well controlled DM was defined when the HbA1c level was < 7%. Agents used in diabetic therapy include the following: sulfonylureas, biguanides, alpha-glucosidase inhibitors, thiazolidinediones, Meglitinide derivatives *etc*[60]. Although effective in reducing blood glucose levels, early sulfonylureas were associated with significant off-target effects, and the biguanide phenformin was discontinued due to adverse events[60]. Although metformin is in the same drug class, it has a better safety profile and is now recommended as first-line treatment. However, many patients require additional glucose control treatment with an agent that has a complementary mechanism of action like metformin. Some common drugs used for treatment of T2DM available in the market include metformin oral, actos oral, Byetta subQ, Janu*via* oral, *etc*.

Another possible way of reversing insulin resistance would be *via* targeting the signaling components in the insulin signaling pathway modulated by HCV. For instance, we have shown that HCV up regulates phospho-S6K1, which stimulates degradation of IRS-1[38]. Thus, targeting phospho-S6K1 would be a target against HCV induced insulin resistance. These studies have not been done yet, so at this time it will be difficult to comment on the predictive outcome on reversal of insulin resistance. Use of specific inhibitors of SOCS-3, which may become useful to correct resistance to both insulin and IFN-α, are not available for clinical use. Alternatively, one may envision inhibiting TNF-α by administering infliximab or similar agents. IR also results from uncontrolled diet and life style. Regulation of weight, diet, and life style management will also be key in managing IR.

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ORIGINAL ARTICLE

Identification and differentiation of PDX1 β-cell progenitors within the human pancreatic epithelium

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Abstract

AIM: To minimize the expansion of pancreatic mesenchymal cells *in vitro* and confirm that β-cell progenitors reside within the pancreatic epithelium.

METHODS: Due to mesenchymal stem cell (MSC) expansion and overgrowth, progenitor cells within the pancreatic epithelium cannot be characterized *in vitro*, though β-cell dedifferentiation and expansion of MSC intermediates *via* epithelial-mesenchymal transition (EMT) may generate β-cell progenitors. Pancreatic epithelial cells from endocrine and non-endocrine tissue were expanded and differentiated in a novel pancreatic epithelial expansion medium supplemented with growth factors known to support epithelial cell growth (dexamethasone, epidermal growth factor, 3,5,3'-triiodo-l-thyronine, bovine brain extract). Cells were also infected with a single and dual lentiviral reporter prior to cell differentiation. Enhanced green fluorescent protein was controlled by the rat *Insulin 1* promoter and the monomeric red fluorescent protein was controlled by the mouse *PDX1* promoter. In combination with lentiviral tracing, cells expanded and differentiated in the pancreatic medium were characterized by flow cytometry (BD fluorescence activated cell sorting), immunostaining and real-time polymerase chain reaction (PCR) (7900HT Fast Realtime PCR System).

RESULTS: In the presence of 10% serum MSCs rapidly expand *in vitro* while the epithelial cell population declines. The percentage of vimentin$^+$ cells increased from 22% ± 5.83% to 80.43% ± 3.24% (14 d) and 99.00% ± 0.0% (21 d), and the percentage of epithelial cells decreased from 74.71% ± 8.34% to 26.57% ± 9.75% (14 d) and 4.00% ± 1.53% (21 d), $P < 0.01$ for all time points. Our novel pancreatic epithelial expansion medium preserved the epithelial cell phenotype and minimized epithelial cell dedifferentiation and EMT. Cells expanded in our epithelial medium contained significantly less mesenchymal cells (vimentin$^+$) compared to controls (44.87% ± 4.93% *vs* 95.67% ± 1.36%; $P < 0.01$). During cell differentiation lentiviral reporting demonstrated that, PDX1$^+$ and insulin$^+$ cells were localized within adherent epithelial cell aggregates compared to controls. Compared to starting islets differentiated cells had at least two fold higher gene expression of PDX1, insulin, PAX4 and RFX ($P < 0.05$).

CONCLUSION: PDX1$^+$ cells were confined to adherent epithelial cell aggregates and not vimentin$^+$ cells (mesenchymal), suggesting that EMT is not a mechanism for generating pancreatic progenitor cells.

Key words: Differentiation; Epithelial; Epithelial-mesenchymal transition; Mesenchymal; PDX1; Insulin; Pro-

genitor; Vimentin

Core tip: Previously, we demonstrated that mesenchymal stem cells could be expanded from human endocrine and non-endocrine pancreas cell fractions *in vitro*. However, we were unable to complete cell differentiation of mesenchymal cell intermediates to functional endocrine cells. In this study we utilized a novel cell culture medium to prevent epithelial cell de-differentiation and mesenchymal cell expansion. After epithelial cell expansion in this medium cells were differentiated *via* our previously described protocol and we confirmed by lineage tracing, flow cytometry, immunostaining and real-time polymerase chain reaction that islet progenitors reside in the pancreatic epithelium and are not derived *via* a mesenchymal cell intermediate.

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INTRODUCTION

Islet transplantation is an attractive alternative to daily insulin injections to achieve a more physiological means for restoring glucose homeostasis[1-3]. Identifying and understanding the origin of a potential human β-cell progenitor could alleviate the current shortage of donor islets and contribute to the overall knowledge of β-cell regeneration. However, the study of β-cell progenitors is fraught with controversy, as several conflicting models and mechanisms describing the origin and existence of these progenitor cells have been proposed. Despite lineage tracing experiments utilizing transgenic mouse models[4-6] the exact origin of β-cell progenitors residing within the pancreas is yet to be elucidated. For example β-cell progenitors have been proposed to originate from: β-cell replication[4], acinar cell transdifferentiation[7,8], ductal cell transdifferentiation[9-12], pancreas derived multipotent precursors[13], pluripotent islet survivor cells[14] and β-cell dedifferentiation with expansion of mesenchymal stem cell (MSC) intermediates *via* epithelial mesenchymal transition (EMT)[15-20].

Previously we reported[21] that MSCs, also referred to as multi-potent stromal cells[22], could be expanded 12-fold from human islet depleted pancreatic tissue (IDPT) that remains following islet isolation. We demonstrated that these pancreatic MSCs could be partially differentiated into islet-like cells. However, in a follow up study[23] we could not restore an epithelial phenotype during tissue culture or generate functional endocrine cells. We hypothesized that this was due in part to our experimental culture conditions, which favored pancreatic MSC expansion and negatively selected pancreatic epithelial cells.

In this study we report that, during *in vitro* pancreatic MSC expansion, epithelial cells also proliferate and when these epithelial cells are enriched and differentiated, this cell population expresses developmental transcription factors indicative of a β-cell progenitor such as PAX4 and RFX6[24-26]. Therefore, to maintain epithelial cell phenotype and allow long-term study of this cell population *in vitro,* we utilized a pancreatic epithelial expansion medium that minimized epithelial cell dedifferentiation and MSC overgrowth in combination with our differentiation protocol[21,23]. Furthermore, by utilizing single and dual lentiviral reporters where, enhanced green fluorescent protein (EGFP) is controlled by the rat *Insulin 1* (Ins1) promoter and monomeric red fluorescent protein (mRFP) is controlled by the mouse *PDX1* promoter[26] we determined that PDX1$^+$ cells observed after 25 d post-differentiation were epithelial cells. Unlike the reversible (EMT) model first described by Gershengorn *et al*[15] and the dedifferentiation of β-cells then replication of β-cell-derived cells described by Russ *et al*[20], we propose that β-cell progenitors reside within the human pancreatic epithelium and that these cells have the potential to respond favorably to *in vitro* differentiation without dedifferentiation into a MSC intermediate *via* EMT. Overall, we report a novel cell culture media that promotes pancreatic epithelial cell survival and minimizes MSC overgrowth, and report that PDX1$^+$ cells observed 25 d post-differentiation are epithelial cells.

MATERIALS AND METHODS

Cell expansion and differentiation

Human islets (n = 9) and IDPT (n = 13) were obtained from the Edmonton Clinical Islet Transplant Program (University of Alberta and Alberta Health Services). Written, informed, consent was provided by donor relatives and all protocols were approved by the UofA Research Ethics Office. Average donor age was 54 (30-71 years) and islet purity assessed by dithizone staining ranged between 10%-40%. IDPT (< 5% insulin positive cells) was obtained following removal of islets by density gradient purification[21,23,27]. Upon receipt, IDPT was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Burlington, ON Canada) supplemented with 0.5% w/v fraction V bovine serum albumin (Sigma-Aldrich, Oakville, ON Canada), 1% insulin-transferrin-selenium (Sigma-Aldrich) and 100 U penicillin/1000 U streptomycin (Invitrogen). Islets were cultured in Connaught Medical Research Laboratories-1066 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 10 mmol/L hydroxyethyl piperazineethanesulfonic acid (HEPES) and 100 U penicillin/1000 U streptomycin (Invitrogen). Both IDPT and islets were cultured in 150 mm non-tissue culture treated plates (Corning, NY, United States) and maintained for 24-48 h at 37 ℃ in 5% CO_2 and 95% air. Following culture, single cell suspensions were derived by, dissociating islets or cellular aggregates derived from the cultured IDPT with 0.05% trypsin, 0.5 mmol/L ethylenediaminetetraacetic acid (Invitrogen) in 1X PBS.

Single cell preparations were cultured and expanded in pancreatic MSC medium or pancreatic epithelial expansion medium. MSC medium is composed of RPMI-1640 supplemented with 10% FBS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate (Invitrogen), 71.5 μmol/L β-mercaptoethanol (Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF, R&D, Minneapolis, MN United States), 20 ng/mL fibroblast growth factor (Invitrogen) and 100 U pencillin/1000 U streptomycin[21,23,27]. Cell confluence was achieved in 10-14 d and cells required passaging every 7 d after that. From both islets and IDPT at the 2nd and 3rd passage we routinely generate a cell population with MSC characteristics as previously described[21,23,27]. To preserve epithelial cell phenotype, single cells derived from islets or the IDPT were also cultured and expanded in a pancreatic epithelial expansion medium either on 150 mm tissue culture treated plates (Corning) or 12 mm poly-l-lysine coated cover slips (BD Biosciences) placed in 24 well tissue culture treated plates (Corning). Pancreatic epithelial expansion medium is composed of Dulbecco's Modified Eagle's Medium/F12 (Invitrogen) supplemented with 0.5% FBS, 0.1 μg/mL EGF, 0.4 μg/mL dexamethasone (Sigma-Aldrich), 14 mg/mL bovine brain extract (Lonza, Walkersville, MD United States), 0.05 μmol/L triiodo-l-thryonine sodium salt (Sigma-Aldrich), 0.1 mg/mL soybean trypsin inhibitor (US Biological, Swampscott, MA, United States), 0.5X ITS+premix (BD Biosciences) and 100 U penicillin/1000 U streptomycin. Expanded cell populations were subsequently differentiated using a multi-step protocol previously described[21,23,27,28] and characterized by flow cytometry, immunohistochemistry and real-time polymerase chain reaction (PCR). For differentiation, the cell monolayer was treated with 20 ng/mL OncostatinM (R&D) for 72 h. In steps 2 and 3 the medium was supplemented with 10 mmol/L nicotinamide (Sigma-Aldrich) for 72 h followed by 10 mmol/L nicotinamide and 10 nmol/L exendin4 (Sigma-Aldrich) for another 72 h. In step 4, 10 ng/mL of transforming growth factor-β1 (TGFβ-1; EMD Millipore, Billerica, MA, United States) was included with nicotinamide and exendin4 for 3-10 d with media changes every 72 h. Cell monolayers were detached with trypsin and aggregated by reconstituting cells at 125000 cells/mL in medium supplemented with nicotinamide, exendin4, TGFβ-1, 0.5X ITS+ premix (BD Biosciences, Beford, MA United States) and transferred to 100 mm ultra-low attachment non-tissue culture treated plates (Corning).

Immunohistochemistry

Double immunofluorescence (IF) analysis was performed on paraffin sections of single cells that had first been fixed with 1% formalin (Fisher Scientific, Nepean, ON Canada) and embedded in 2% low melting point agarose (Sigma-Aldrich) or cells which had been differentiated on 12 mm poly-l-lysine cover slips (BD Biosciences). Paraffin sections were processed and immunostained as previously described[23,28]. Cover slips were fixed in 1% formalin for 30 min in the dark at 4 ℃, and then washed twice with 5% normal goat serum (NGS) in PBS. For antibodies requiring permeabilization, 0.3% saponin (Sigma) in PBS was applied for one minute, and another two washes of 5% NGS followed. All cover slips were then blocked with 20% NGS for 1 h in the dark. Primary antibodies were diluted in 5% NGS at the following concentrations: 1/200 anti-epithelial cell adhesion molecule (EpCAM, Stem Cell Technologies, Vancouver, BC Canada), 1/100 anti-vimentin (Dako, Mississauga, ON Canada), 1/25 anti-human proliferating cell nuclear antigen (PCNA, Invitrogen), 1/50 anti-CK19 (Dako), 1/5000 anti-glucagon (Sigma-Aldrich), 1/1000 anti-insulin (Dako), 1/1000 anti-pancreatic polypeptide (Dako), 1/1000 anti-somatostatin (Dako), 1/1000 anti-PDX1 (Abcam, Cambridge, MA, United States). All appropriate species-specific secondary antibodies were AlexaFluor 488 or 594 conjugates (Molecular Probes, Eugene, OR, United States) and diluted 1/200 in 5% NGS. Slides and or cover slips were cover-slipped with ProLong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (Invitrogen) to counter stain nuclei and preserve fluorescence. Negative controls were incubated without primary antibodies and positive controls were sections of normal human and infant pancreas. All slides were visualized with an Axioscope Ⅱ equipped with AxioCam MRC and analyzed with Axiovision 4.6 (Carl Zeiss, Gottingen, Germany).

Flow cytometry

Single cells from islets and IDPT were stained and analyzed by fluorescence activated cell sorting (FACS) using the FACS Calibur (BD Biosciences) and Cell Quest Pro software and compared to matched isotype controls[23,27]. Cells were permeabilized with 0.3% saponin for 60 min and stained with 1/10 vimentin-FITC, 1/5 EpCAM-FITC and 1/15 PCNA-647 (BioLegend, San Diego, CA, United States). Isotype controls were; IgG1-FITC, IgG2a-FITC and IgG2a-647. Values are expressed as mean percent ± SE.

RNA isolation and real-time PCR

Islets and IDPT cells prior to cell culture, during expansion and post differentiation were preserved in Trizol reagent (Invitrogen) and stored at -80 ℃. RNA was extracted in combination with the RNeasy Mini Kit (Qiagen, Mississauga, ON Canada) as per the manufacturer's protocol. cDNA was synthesized as described[21]. Real-time PCR was performed using the "TaqMan gene expression assay" (Applied Biosystems, Invitrogen) and a 7900HT Fast Realtime PCR System (Applied Biosystems). Relative quantification was performed by utilizing the comparative Ct method and all results were compared to the control samples for each time point after normalizing to an endogenous control (beta 2-microglobulin) using the Relative Quantification Manager software (Applied Biosystems). Values are expressed as mean percent ± SE. cDNA negative controls contained water in place of RNA and RT-PCR negative controls contained water in place of cDNA, β2-microglobulin (β2m) ensured cDNA integrity.

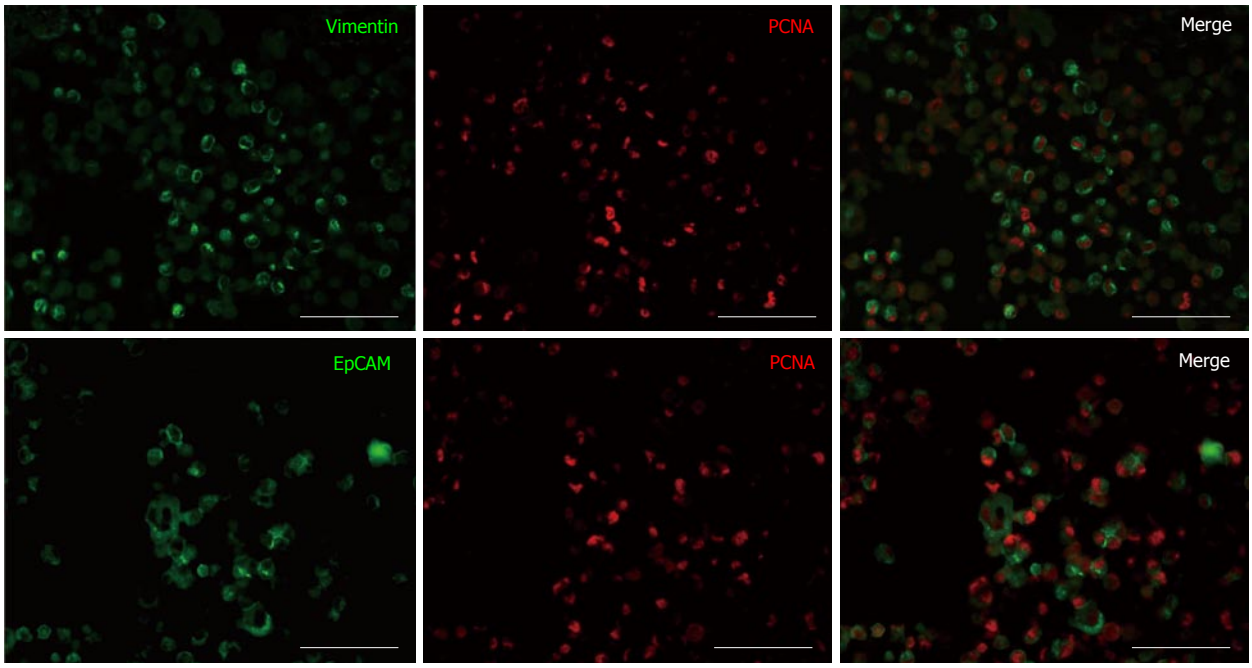

Figure 1 Double immunofluorescence staining of islet depleted pancreatic tissue expanded cells after 14 d in culture. Cells are positive for vimentin (green), EpCAM (green) and PCNA (red). Both vimentin and EpCAM positive cells are positive for PCNA staining (merged) and proliferating. Scale bars are 50 μm. EpCAM: Epithelial cell adhesion molecule; PCNA: Proliferating cell nuclear antigen.

Lentivirus infection

Lentiviral vectors were kindly provided by Dr. James D. Johnson (University of British Columbia, Vancouver, BC, Canada) and described in detail by Szabat *et al*[26]. We received the following vectors: dual reporter mouse PDX1 promoter-mRFP/rat Ins1 promoter-EGFP, single reporter mouse PDX1 promoter-mRFP, single reporter rat Ins1 promoter-EGFP as well as the structural and envelope vectors. Virus was produced by transfection of 293T cells that were a gift from Dr. Patrick MacDonald (University of Alberta, Edmonton, AB, Canada) utilizing FuGENE6 Transfection Reagent (Roche Diagnostics, IN, United States) and the protocol first described by Dr. Garry Nolan Lab (http://www.stanford.edu/group/nolan/index.html). Virus was titred using the rat INS1 cell line (a gift from Dr. Patrick MacDonald) and titres were between 2-4 × 10^6 TU/mL with an infection efficiency of 40%-70%. Single cells from human islets or IDPT were plated at a density of 0.3 × 10^6 cells/well onto a 24 well plate that contained 12 mm poly-l-lysine cover slips and cultured in pancreatic epithelial expansion medium. Cells were allowed to adhere and infected at a multiplicity of infection of < 1. Protein expression (fluorescence) was monitored daily and peak fluorescence of human primary cells was routinely detected between 4-7 d post infection. Differentiation of infected primary cells was started at 4-7 d post-infection. Absolute counts of positive mRFP and EGFP cells were counted using ImageJ software[29].

Statistical analysis

Data is expressed as mean ± SE. Statistical comparisons were performed with STATA11 (StataCorp LP, College Station, TX) using one-way analysis of variance and Bonferroni post-hoc test. Acceptable level of significance was considered $P < 0.05$.

RESULTS

Epithelial cells from IDPT proliferate in pancreatic MSC medium

Previously, we demonstrated that during *in vitro* cell expansion of cells from IDPT, the proportion of epithelial cells decreased while vimentin positive cells (MSCs) significantly increased[21,23]. In this study, to determine if epithelial cells were still capable of proliferation, we assessed cell proliferation after 14 d in culture *via* dual IF staining and flow cytometry. Cells that were formalin fixed and embedded in agarose were stained with antibodies against EpCAM or vimentin (MSC) then co-stained for PCNA. After 14 d in culture we confirmed that vimentin$^+$ cells were the predominant cell population and these cells were PCNA$^+$ and proliferating (Figure 1). A small proportion of epithelial cells (EpCAM$^+$) were still present and were also PCNA$^+$ thus still proliferating (Figure 1). The proportion of vimentin and EpCAM cells that were positive for PCNA were quantified by FACS analysis at Time 0, 14 and 21 d in culture (Table 1). During cell expansion; the percentage of vimentin$^+$ cells proliferating (PCNA$^+$) increased from 22% ± 5.83% to 80.43% ± 3.24% (14 d) and 99.00% ± 0.0% (21 d), and the percentage of proliferating epithelial cells decreased from 74.71% ± 8.34% to 26.57% ± 9.75% (14 d) and 4.00% ± 1.53% (21 d), $P < 0.01$ for all time points. Therefore, MSC expansion culture conditions favor MSC expansion over epithelial cells

Table 1 Cell composition and proportion of proliferating cell nuclear antigen positive cells during pancreatic cell expansion from islet depleted pancreatic tissue

Days in culture	Percent positive				
	Vimentin	EpCAM	PCNA	Vim/ PCNA	EpCAM/ PCNA
0 (n = 7)	22.71 ± 4.93	87.57 ± 3.96	73.57 ± 8.42	22.00 ± 5.83	74.71 ± 8.34
14 (n = 7)	80.29 ± 3.07[b]	23.69 ± 7.88[b]	88.43 ± 2.91	80.43 ± 3.24[b]	26.57 ± 9.75[b]
21 (n = 3)	99.00 ± 0.0[b]	3.33 ± 0.88[b]	99.00 ± 0.0	99.00 ± 0.0[b]	4.00 ± 1.53[b]

Data represent means ± SE. Statistical analysis of differences between the groups was performed with STATA11 (StataCorp LP, College Station, TX) using one-way analysis of variance and Bonferroni post-hoc test. [b]$P < 0.01$ compared to Time 0. EpCAM: Epithelial cell adhesion molecule; PCNA: Proliferating cell nuclear antigen.

Table 2 Preservation of epithelial cells during culture in a defined epithelial medium

Condition	Percent positive	
	Vimentin	EpCAM
Epithelial Medium (n = 7)	44.87 ± 4.93[b]	24.10 ± 8.60
MSC Medium (n = 3)	95.67 ± 1.36[b]	17.43 ± 6.88

Data represent means ± SE. Statistical analysis of the differences between the groups was calculated with STATA11 (StataCorp LP, College Station, TX) using one-way analysis of variance and Bonferroni post-hoc test. [b]$P < 0.01$ for vimentin positive cells expanded in epithelial medium *vs* MSC medium. EpCAM: Epithelial cell adhesion molecule; MSC: Mesenchymal stem cell.

Epithelial cell phenotype is preserved when IDPT is cultured in defined pancreatic epithelial expansion medium

Our MSC expansion medium limits pancreatic epithelial cell growth *in vitro*[21,23,27]. We determined that sorted epithelial cells could expand and divide in MSC medium but vimentin$^+$ MSCs were still the predominating cell population (not shown). By reducing the FBS content from 10% to 0.5% and including growth factors known to promote epithelial cell survival under reduced serum conditions such as EGF, 3,5,3'-triiodo-l-thyronine and bovine brain extract[30-32] we were able to minimize MSC overgrowth and preserve the epithelial cell phenotype (Table 2). IDPT cells cultured in pancreatic epithelial expansion medium contained significantly less vimentin$^+$ cells (44.87% ± 4.93%) than cells expanded in MSC expansion medium (95.67% ± 1.36%; $P < 0.01$). Therefore, pancreatic epithelial expansion medium was used to trace cell fate and was supplemented for cell differentiation.

Culture in differentiation medium increases the proportion of PDX1 and insulin positive cells

To determine the progenitor cell content within the pancreatic epithelium, cells from dissociated islets and IDPT were seeded onto poly-l-lysine coated cover slips placed in 24 well plates with pancreatic epithelial expansion medium. Prior to differentiation cells were infected with, the PDX1-mRFP-Ins1-EGFP dual reporter, PDX1-mRFP or Ins1-EGFP single reporter lentivirus then characterized *via* IF staining and real-time PCR. Fluorescence was detected between 4-7 d post infection in both islet and IDPT preparations, at which time differentiation was initiated. Controls were infected but not differentiated. During islet cell differentiation, cell aggregates formed throughout the cell monolayer. Within these adherent aggregates PDX1$^+$ (RFP) and insulin$^+$ (EGFP) expressing cells were observed (Figure 2A). In addition, both single positive (PDX1$^+$ or INS$^+$) and double positive (PDX1$^+$ INS$^+$) cells were observed (Figure 2A). In undifferentiated conditions fewer positive cells and cell aggregates were observed (Figure 2B). When analyzing image fields, in 4/4 cell preparations approximately twice as many PDX1$^+$ and insulin$^+$ cells were observed in differentiated conditions (51.25 ± 17.24 mRFP and 28.13 ± 9.34 EGFP) versus undifferentiated conditions (31.75 ± 10.83 mRFP and 14.50 ± 4.03 EGFP) as determined by absolute cell counts. Relative quantification of gene expression by real-time PCR (Figure 2C) confirmed this observation and demonstrated that differentiated islet cells compared to starting islets had increased expression of PDX1 ($P < 0.05$), insulin ($P < 0.01$) PAX4 ($P < 0.05$) and RFX6 ($P < 0.01$). A similar pattern was observed in differentiated (Figure 3A) and undifferentiated (Figure 3B) wells of cultured IDPT infected with PDX1-mRFP or PDX1-mRFP-Ins1-EGFP, although much less PDX1 and insulin expression (not shown) was observed compared to islet cell cultures.

PDX1 progenitor cells are localized within the pancreatic epithelium

To identify cells within the differentiated cell aggregates that were PDX1$^+$, and to confirm expression after lentiviral infection, cells were characterized by IF staining (Figures 4 and 5) utilizing the following antibodies: EpCAM, vimentin, CK19, PDX1, glucagon, insulin, pancreatic polypeptide and somatostatin. Cells positive for glucagon, pancreatic polypeptide and somatostatin, still remained after 25 d in culture compared to controls although these cells were infrequent and did not express PDX1$^+$ (not shown). PDX1$^+$ cells were shown to be negative for vimentin staining (Figure 4) and CK19 (not shown). PDX1$^+$ cells did however, co-stain with EpCAM (Figure 4) demonstrating that in our experimental conditions PDX1$^+$ cells are localized to the epithelial cell population. PDX1 and insulin expression was verified by, insulin and PDX1 primary antibody staining (Figure 5). Nuclear and cytoplasmic PDX1 staining (Figure 5) was observed while insulin staining was confined to the cytoplasm (Figure 5).

DISCUSSION

Although several recent human studies that involve lineage tracing[13,14,20,26] have been conducted, the debate continues as to the origin of human β-cell progenitors. Sev-

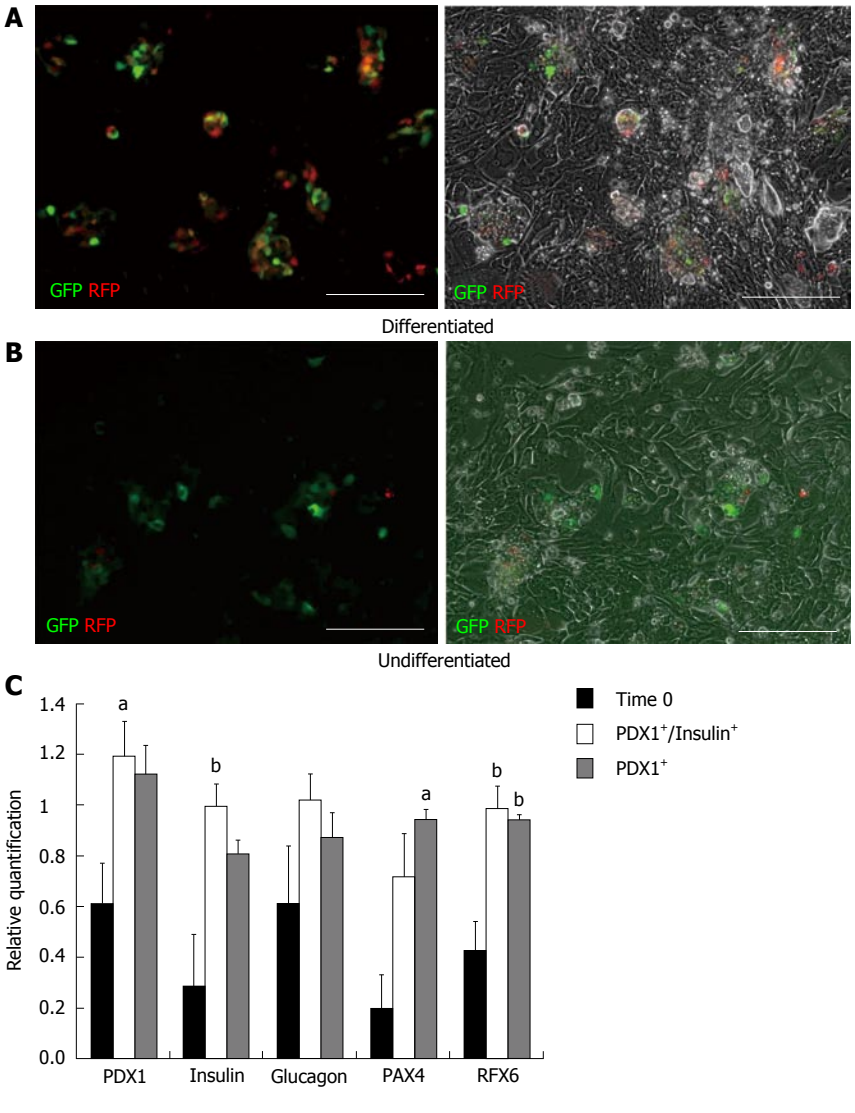

Figure 2 Comparison of differentiated and undifferentiated islet cells infected with PDX1-monomeric red fluorescent protein/insulin 1-enhanced green fluorescent protein. Islet cells cultured in differentiation medium (A) form adherent cell aggregates within the cell monolayer and insulin$^+$ (GFP) and PDX1$^+$ (RFP) expressing cells are localized within these cell aggregates. Islet cells cultured in control medium (B, undifferentiated) have fewer cell aggregates and insulin (GFP) and PDX1$^+$ (RFP) cells. Scale bars are 100 μm. Gene expression (C) of PDX1-monomeric red fluorescent protein (mRFP)/insulin-enhanced green fluorescent protein islet cells (PDX1$^+$ insulin$^+$, white bars; n = 7) and PDX1-mRFP infected islet cells (PDX1$^+$, grey bars; n = 4) post-differentiation compared to starting islet tissue (Time 0, black bars; n = 8) measured by real-time PCR. $^aP < 0.05$ and $^bP < 0.01$ compared to Time 0. RFP: Red fluorescent protein.

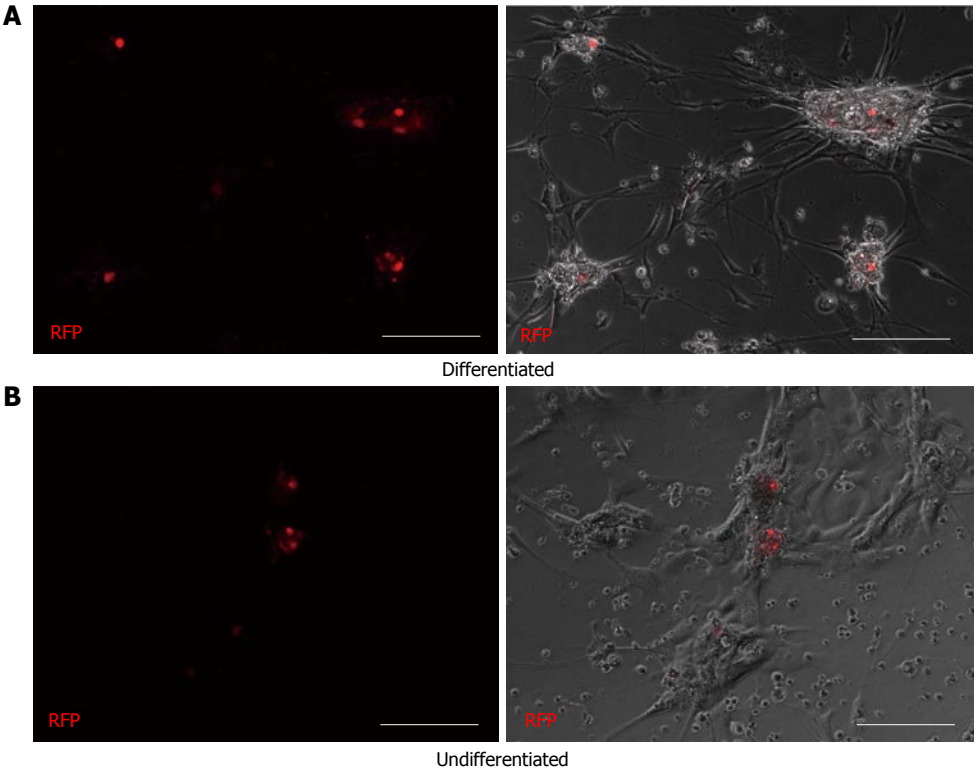

Figure 3 Comparison of differentiated (A) and undifferentiated (B) cells from the islet depleted pancreatic tissue infected with PDX1-monomeric red fluorescent protein. A few PDX1$^+$ cells (RFP) are visible within the adhered aggregates in the differentiated condition (A). Cell aggregates are absent in the undifferentiated cell conditions (B) and PDX1$^+$ cells are within the monolayer. Scale bars are 100 μm. RFP: Red fluorescent protein.

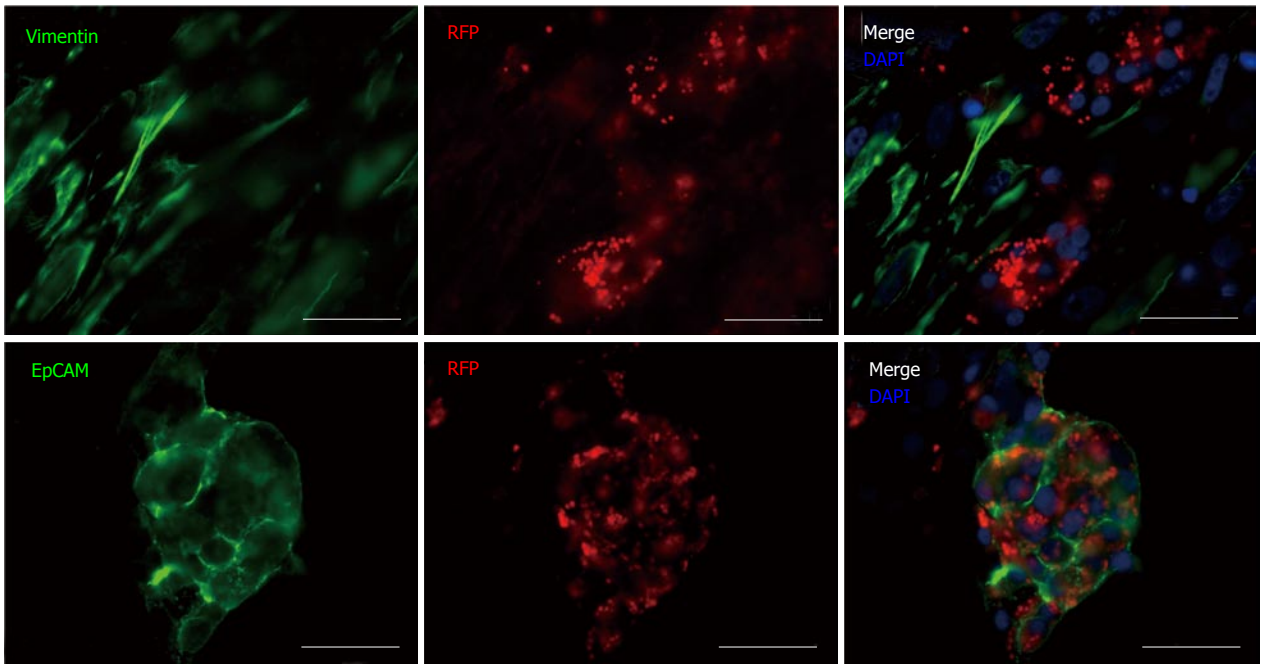

Figure 4 Immunofluorescence staining of differentiated PDX1- monomeric red fluorescent protein infected islet cells with primary antibodies to vimentin and epithelial cell adhesion molecule, with secondary antibodies conjugated to Alexa-488 (green). PDX1^{+} positive cells (RFP) are not co-localized with vimentin (merged), but are co-localized with epithelial cell adhesion molecule. Nuclei are stained blue with DAPI. Scale bars are 20 μm. RFP: Red fluorescent protein; DAPI: 4',6-diamidino-2-phenylindole.

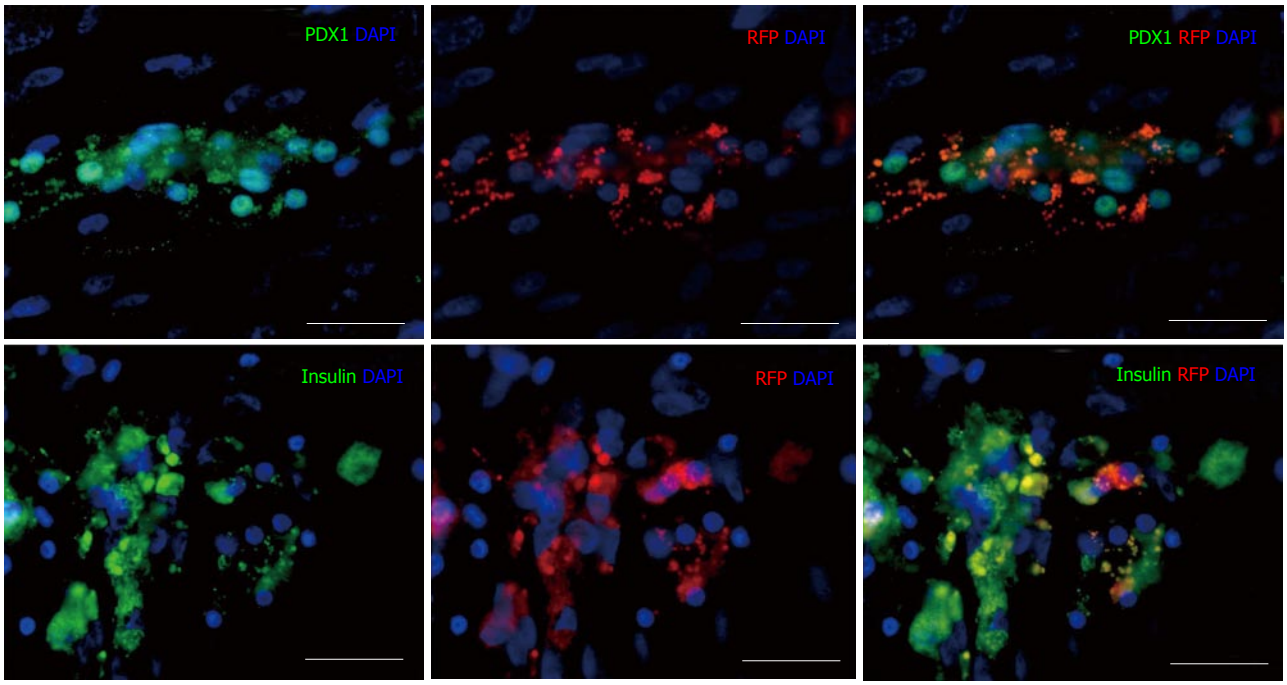

Figure 5 Immunofluorescence staining of differentiated PDX1-monomeric red fluorescent protein infected islets with primary PDX1 and insulin antibodies with secondary antibodies conjugated to Alexa-Fluor488 (green). PDX1^{+} cells (RFP) nuclei stain positive with PDX1/Alexa-488 antibody confirming lentiviral expression. Insulin/Alexa-Fluor488 (green) stains insulin within PDX1^{+} infected cells (yellow). Nuclei are stained blue with DAPI. Scale bars are 20 μm. RFP: Red fluorescent protein.

eral studies have proposed that β-cell progenitors can be derived from a mesenchymal cell intermediate *via* EMT. In our previous studies we were also able to expand MSCs from exocrine and endocrine cell explants[21,23]. We determined that when the culture medium contained 10% serum, pancreatic MSCs could be rapidly expanded from pancreatic cells and that these MSCs could be easily differentiated into mesoderm (bone, fat and cartilage)[21,28]. However, in those studies pancreatic MSCs could only be partially differentiated into endocrine cells and we were

unable to restore the epithelial cell phenotype or detect insulin protein by immunostaining[21,23]. We have since hypothesized that in our differentiation conditions it is the low percentage of epithelial cells, which remain after mesenchymal cell expansion, that respond favorably to our differentiation protocol[21,23] and not MSCs or a MSC intermediate. Thus, the dedifferentiation of islet or IDPT cells during long-term culture results in the loss of epithelial cells thus making this population difficult to follow *in vitro*.

In this study we utilized a reduced serum medium supplemented with growth factors known to support epithelial cells (EGF, 3,5,3'-triiodo-l-thyronine, bovine brain extract) and were able to minimize EMT and preserve the epithelial phenotype (Table 2). We determined that by preventing cell dedifferentiation and MSC overgrowth we could maintain the epithelial phenotype for greater than 25 d. If in fact epithelial mesenchymal transition[15,20] is a necessary process where progenitors or β-cells must dedifferentiate to replicate and then be redifferentiated into insulin producing cells then an alternate cell culture model must be employed. However, it is unclear if dedifferentiation, expansion then redifferentiation is a preferential model for increasing β-cells since β-cells generated in this model do not secrete physiologic levels of insulin compared to normal β-cells[15,20].

Lentiviral tracing[26] in combination with our pancreatic epithelial expansion medium, allowed us for the first time to observe morphological changes during *in vitro* differentiation without MSC overgrowth. Compared to lentiviral infected controls (undifferentiated) we have concluded that it is within the adherent differentiated cellular aggregates where PDX1$^+$ and insulin$^+$ cells reside. In addition, it is within these cellular aggregates where epithelial cells (EpCAM) that are PDX1$^+$ are located. More importantly we did not observe vimentin$^+$ or ductal epithelial cells (CK19$^+$) that were PDX1 or insulin positive within the cell aggregates. Although several citations have demonstrated that pancreatic ductal epithelial cells can generate new β-cells[9-12], we did not observe this in our differentiation model. In addition, the infrequent cells we observed that were pancreatic polypeptide, somatostatin, and glucagon positive were also negative for PDX1 and insulin. By relative quantification we observed increased gene expression of developmental transcription factors indicative of a β-cell progenitor[24-26]. Differentiated cells compared to starting islets had at least two fold higher expression of PDX1, insulin, PAX4 and RFX (Figure 2C).

In summary we describe a unique cell culture condition for long-term study of pancreatic epithelial progenitor cells that minimizes overgrowth of MSCs (vimentin$^+$) and dedifferentiation of epithelial cells through EMT. We confirmed that during differentiation *via* lentiviral reporting that PDX1$^+$ cells were confined to epithelial cell aggregates that form during differentiation and not vimentin$^+$ cells suggesting that EMT is not a mechanism for generating pancreatic progenitor cells. Future studies will be to determine overall function of differentiated cells.

ACKNOWLEDGMENTS

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COMMENTS

Background

Islet transplantation is an attractive alternative to daily insulin injections. Identifying and understanding the origin of a potential human β-cell progenitor could alleviate the current shortage of donor islets and contribute to the overall knowledge of β-cell regeneration. However, the study of β-cell progenitors is fraught with controversy, as several conflicting models and mechanisms describing the origin and existence of these progenitor cells have been proposed.

Research frontiers

A popular model describing the origin of human β-cell progenitors that has been proposed is β-cell dedifferentiation and the expansion of a mesenchymal stem cell (MSC) intermediate *via* epithelial-mesenchymal transition (EMT). However, there has been limited success when redifferentiating these mesenchymal cells back into functional β-cells.

Innovations and breakthroughs

The authors previously demonstrated that MSCs, could be expanded 12-fold from human islet depleted pancreatic tissue that remained following islet isolation and demonstrated that these pancreatic MSCs could be partially differentiated into islet-like cells *in vitro*. However, in a follow up study the authors could not restore an epithelial phenotype or generate functional endocrine cells. The authors determined that the few remaining epithelial cells after MSC expansion were the cells that responded to the authors differentiation protocol. In these culture conditions MSC overgrowth prevented pancreatic epithelial progenitor expansion and differentiation. In this study the authors report that by using a novel pancreatic epithelial medium, MSC expansion and epithelial cell dedifferentiation can be minimized and pancreatic epithelial cell progenitors can be successfully expanded and differentiated.

Applications

This study demonstrates that β-cell progenitors reside in the pancreatic epithelium and that EMT should be inhibited *in vitro* to successfully expand and differentiate these progenitors. The authors conclude that EMT is not a mechanism for generating pancreatic progenitor cells.

Terminology

EMT occurs *in vitro* when epithelial cells de-differentiate and lose their phenotype. The resulting cells are MSCs otherwise known as multi-potent stromal cells.

Peer review

The authors describe a new method that could in the future allow for *in vitro* generation of insulin-producing cells. The work is well done and appropriately presented.

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BRIEF ARTICLE

Starting glargine in insulin-naïve type 2 diabetic patients based on body mass index is safe

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Abstract

AIM: To evaluate the safety of four insulin titration algorithms in a homogeneous population of insulin-naïve type 2 diabetic patients.

METHODS: We conducted a 24-wk, open, single-center study with 92 insulin-naïve type 2 diabetes patients who failed treatment with one or two oral drugs. The patients were randomized to one of the four following algorithms: LANMET (n = 26) and LANMET PLUS (n = 22) algorithms, whose patients received a fixed initial insulin dose of 10 U, and DeGold (n = 23) and DeGold PLUS (n = 21) algorithms, whose patients' initial insulin dose was based on their body mass index (BMI). In addition, patients in the PLUS groups had their insulin titrated twice a week from 2 to 8 U. In the other two groups, the titration was also performed also twice a week, but in a fixed increments of 2 U. The target fasting glucose levels for both groups was 100 mg/dL.

RESULTS: There was no significant difference in efficacy parameters. There was no significant difference when comparing moderate hypoglycemia events in algorithms starting with a 10 U fixed dose and algorithms based on BMI. However, there was a significant increase in moderate hypoglycemia events among the PLUS treated patients when the LANMET and DeGold algorithms were compared with the 2 fast-titration PLUS algorithms. We observed 12 hypoglycemia events in the first group, which corresponded to 0.94 events/patient per year, and we observed 42 events in the second group, which corresponded to 2.81 events/patient per year (P < 0.037). No further significant differences were observed when other comparisons between the algorithms were carried out.

CONCLUSION: Starting insulin glargine based on BMI is safe, but fast titration algorithms increase the risk of moderate hypoglycemia.

Key words: Type 2 diabetes; Insulin glargine; Basal insulin; Hypoglycemia; Titration algorithms

Core tip: To start insulin therapy in insulin naïve type 2 diabetes patients, a long-acting basal insulin, such as insulin glargine, is added once a day. The majority of algorithms determine insulin titration according to fasting plasma glucose levels, but the dosage differs at the initial dose, frequency and speed of adjustments. It is difficult to compare the different algorithms employed in trials with populations of different socio-economic strata and variable access to educational materials. Here, we compared the safety of different titration algorithms in a population that was homogeneous in terms of socio-economic strata and with the same degree of education in diabetes.

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INTRODUCTION

Type 2 diabetes is characterized by insulin resistance and is associated with the incremental loss of pancreatic beta cell mass and/or function[1]. Patients who are initially capable of maintaining a good metabolic control using oral anti-diabetes drugs (OADs) frequently need to add insulin to their treatment over time[2]. The simplest way to begin insulin therapy is to add a long-acting basal insulin, such as insulin glargine, once per day[3].

Basal insulin therapy is an efficient glycemia-lowering treatment, provided it is delivered in the appropriate doses. Therefore, it must be carefully titrated until patients achieve the established fasting plasma glucose goal (FPG)[4]. Several titration algorithms have been validated in clinical trials, and they can be used to guide basal insulin dose adjustments. Most algorithms determine insulin titration according to FPG levels, but differ in the initial insulin dose, frequency, and speed of dose adjustments[5-7]. A new algorithm (DeGold) has been recently described, and it considers the degree of insulin resistance due to obesity and recommends initial doses ranging from 0.2 to 0.35 U/kg according to the patient's body mass index (BMI)[8].

The initial insulin dose is important for predicting whether a target can be reached and how long titration will take[4] before treatment is started. Treatment compliance may be jeopardized if the treatment period is too long and if patients do not see any significant changes in their FPG levels. The frequency and speed at which insulin doses are adjusted also vary according to the chosen algorithm. For example, in the AT.LANTUS trial with insulin Glargine, titration from 2 to 8 U weekly according to the FPG that was performed by physicians was compared to the increment of 2 U every 3 d until the FPG reached 100 mg/dL that was performed by the patients themselves. The results showed that titration performed by patients could be more effective in achieving A1C targets[6]. In the Canadian INSIGHT Trial, patients titrated their insulin Glargine dose by adding 1 U/d until they reached the target of 100 mg/dL FPG[5].

Provided it is employed correctly according to the "Treat to Target" concept, any algorithm can bring fasting glucose levels to normal and allow patients who are not in need of additional prandial therapy, like rapid acting insulin, to achieve the desired glycated hemoglobin (A1C) values[7].

Hypoglycemia may also be a factor in the achievement of a glycemic target. The occurrence of hypoglycemia events is not solely due to the effects caused by exogenous insulin[9] but is also fundamentally linked to other factors, including the level of education of diabetic patients, especially in regard to compliance to treatment and protective measures against hypoglycemia[10]. It is difficult to compare the efficacy and the safety of all different algorithms used in trials that have populations belonging to different socioeconomic levels and having different access to educative measures[4,7,10]. As such, we decided to compare the safety of different titration algorithms in a population that was homogeneous in terms of socioeconomic level and level of education in diabetes.

The main objective of the study was to evaluate the safety of four insulin glargine titration algorithms applied to a homogeneous sample of insulin-naïve type 2 diabetes patients and to compare the frequency of severe and moderate hypoglycemia (glycaemia < 56 mg/dL) events, the frequency of nocturnal symptomatic hypoglycemia, total number of hypoglycemic events, and serious adverse events. The efficacy parameters analyzed for each algorithm were the changes in A1C from baseline to study end, changes in FPG levels, weight variation during the study, insulin doses, time needed to reach the FPG target, and the proportion of patients who reached an A1C target between 7% and 7.5%, and below 7%.

MATERIALS AND METHODS

Population sample and experimental design

This was a 24-wk, single-centered, randomized, open study. We screened 125 patients diagnosed with type 2 diabetes, > 18 years old and BMI < 40 kg/m^2 who had been on stable treatment with one or two OADs for more than 3 mo, and A1C between 7% and 12%. The main criteria for exclusion were as follows: chronic kidney disease, liver disease with transaminases ⩾ 2.5 times the normal value, and any pathology requiring systemic corticosteroid treatment. A total of 33 patients were excluded because their A1C was above threshold, their hepatic enzymes were above normal, or they had moderate renal failure.

The study was approved by the local institucional review board and was conducted according to the Helsinki Declaration and the GCP-ICH. Informed consent was obtained from all patients. All patients were living in the area outside of São Paulo city, had the same socioeconomic background and were insulin treatment naïve. All patients attended the same education sessions on diabetes, and lessons were always given by the same person.

Comparisons between the algorithms were made using ANOVA/Kruskal-Wallis and Student's *t* tests. The data on patients who completed the protocol were used, and all patients who received at least one dose of insulin to evaluate data on safety parameters were included.

The demographic data of the randomized patients are shown on Table 1. Population homogeneity was tested and showed the groups were similar in terms of age, weight, BMI, time they have had diabetes for, initial A1C level, and previous treatment with OADs. However, the proportion of M/F gender was significantly different in the LANMET PLUS ($P < 0.047$) group.

After 4 wk of a run-in period, 92 patients were ran-

Table 1 Characteristics of the patient population, as grouped according to the four algorithms

	n	Gender	Age (yr)	Weight (kg)	BMI (kg/m^2)	Duration (yr)	Baseline A1C	Baseline FPG (mg/dL)	Previous treatment
Fixed titration 2/2 U									
LANMET	26	8 M	55.0 ± 10	78 ± 16.6	30.7 ± 4.95	8 ± 4.23	9.39% ± 1.67%	193.0 ± 59.4	2 OAD (20) 1 OAD (6)
Variable titration									
LANMET PLUS	22	6 M	52.3 ± 7.7	70.6 ± 13	27.8 ± 4.7	7.8 ± 3.8	9.35% ± 1.34%	179.4 ± 51.4	2 OAD (21) 1 OAD (1)
Fixed titration 2/2 U									
DeGold	23	14 M	54.6 ± 8	78.3 ± 13.5	28.8 ± 4.4	10.2 ± 7.1	9.21% ± 1.30%	196.6 ± 54.8	2 OAD (19) 1 OAD (4)
Variable titration									
DeGold PLUS	21	12 M	53.8 ± 7.6	79.3 ± 15.9	29.5 ± 4.4	9.8 ± 5.4	9.61% ± 1.69%	196.1 ± 53.4	2 OAD (19) 1 OAD (2)

OAD: Oral anti-diabetes drug; FPG: Fasting plasma glucose; BMI: Body mass index.

Table 2 Treatment algorithms used in this study

Initial dose	BMI	Algorithms			
		LANMET fixed	LANMETPlus	DeGold fixed	DeGoldPlus
Fixed initial dose in U	n.a.	10		10	
Variable dose according to BMI (kg/m^2) in U/kg	< 26		0.2		0.2
	26 < 30		0.25		0.25
	30 < 35		0.3		0.3
	> 35		0.35		0.35
Insulin adjustment	FPG				
Fixed Titration twice/week in U		2	2		
Variable titration according to FPG (mg/dL) twice/week in U	< 100			0	0
	101 < 120			-2	-2
	121 < 140			2	2
	141 < 180			4	4
	> 180			-2	-2

FPG: Fasting plasma glucose; BMI: Body mass index.

domly distributed to the four algorithms and were treated for the next 16 wk. During this period, 10 visits were scheduled and telephone monitoring was performed by the investigators between visits. A follow-up visit was performed 4 wk after the completion of the study. Three patients withdrew their informed consent. No patients dropped out due to hypoglycemia or any other adverse events.

Most patients were being treated with metformin and sulfonylurea, except for one patient in the DeGold PLUS group who received nateglinide and metformin, and another one in the LANMET PLUS group who received rosiglitazone and metformin. Thirteen patients were on monotherapy, of which seven were on sulfonylurea and six were on metformin. All patients were kept solely either on metformin 2 g/d or on the maximum tolerated dose during the treatment period.

Treatment algorithms

LANMET and LANMET PLUS used the same initial Insulin Glargine dose of 10 U, while DeGold and DeGold PLUS used an initial insulin Glargine dose based on BMI, as shown on Table 2. For the LANMET and DeGold algorithms, the insulin doses were increased by 2 U, twice a week, to reach the FPG target of 100 mg/dL. For LANMET Plus and DeGold Plus, titration was performed by increasing insulin doses, from 2 to 8 U total, twice a week, according to the FPG.

Patients administered the insulin at bedtime and adjusted the doses under the supervision of a person over the phone. In all algorithms, the titration of insulin doses was delayed and an immediate reduction of the insulin dose was recommended if hypoglycemia < 70 mg/dL. Insulin titration continued in all algorithms until the targeted FPG, which was between 80 and 100 mg/dL, was reached. The insulin dose was then maintained and considered adequate when at least 50% of the subsequent FPG measurements corresponded to the aimed target.

Rescue therapy with rapid acting insulin was used on one patient who presented with persistent A1C > 8%, even though he had his FPG on target for more than 6 wk.

The patients measured their capillary FPG daily and were instructed to repeat the measurements if they started having symptoms suggestive of hypoglycemia. When necessary, the mean values of 3 d of capillary FPG were used to calculate a new insulin dose.

Classification of hypoglycemia

Severe hypoglycemia: Severe hypoglycemia was defined as an event requiring third party assistance and glucose levels below 30 mg/dL, or if the patient recovered after receiving oral carbohydrates, intravenous glucose, or glucagon.

Symptomatic hypoglycemia: Symptomatic hypoglycemia was defined as an event where the patient presented with symptoms of hypoglycemia, but responded to oral carbohydrate ingestion or had a glycemia < 70 mg/dL (mild) or < 56 mg/dL (moderate).

Asymptomatic hypoglycemia: Asymptomatic hypoglycemia was defined as an event without any hypoglycemia symptoms, but glucose levels below 70 mg/dL.

Asymptomatic nocturnal hypoglycemia: Asymptomatic nocturnal hypoglycemia was determined when glycemia under 70 mg/dL was detected before breakfast.

Table 3 Treatment efficacy data *n* (%)

	LANMET	LANMET PLUS	DeGold	DeGold PLUS
Initial insulin dose (U)	10.0 ± 0	10.0 ± 0	21.0 ± 7.3	18.3 ± 7.0
Initial insulin dose (U/kg)	0.13 ± 0.02	0.13 ± 0.03	0.26 ± 0.05	0.25 ± 0.05
Final insulin dose (U)	41.65 ± 14.00	87.00 ± 26.87	54.68 ± 21.63	48.19 ± 38.50
Final insulin dose (U/kg)	0.54 ± 0.20	0.59% ± 0.27%	0.67% ± 0.24%	0.65% ± 0.52%
Baseline A1C	9.39% ± 1.67%	9.35% ± 1.34%	9.21% ± 1.30%	9.61% ± 1.69%
Final A1C	7.36% ± 1.32%	7.32% ± 0.67%	6.82% ± 0.70%	7.38% ± 0.95%
Reduction in A1C	2.02% ± 1.60%	2.02% ± 1.17%	2.48% ± 1.23%	2.23% ± 1.69%
Proportion of patients reaching FPG target	19/26 (73)	16/20 (80)	22/23 (95)	20/21 (95)
Proportion of patients reaching A1C ⩽ 7.5%	17/26 (65)	13/20 (65)	20/23 (87)	13/21 (62)
Proportion of patients reaching A1C ⩽ 7.0%	11/26 (42)	5/20 (25)	16/23 (69)	7/21 (33)
Duration of titration to reach FPG target (d)	28 ± 31	15 ± 19	22 ± 20	20 ± 17
Weight variation (kg)	0.276 ± 2.94	1.190 ± 2.430	0.954 ± 2.590	1.630 ± 2.500
Final FPG (mg/dL)	119.4 ± 36.2	109.0 ± 28.7	106.6 ± 18.0	107.6 ± 17.3

FPG: Fasting plasma glucose.

Table 4 Hypoglycemia events *n* (%)

	LANMET	LANMET PLUS	DeGold	DeGold PLUS	LANMET and LANMET PLUS	DeGold and DeGold PLUS	LANMET and DeGold	LANMET PLUS and DeGold PLUS
					Fixed initial dose	Variable initial dose	Fixed titration	Variable titration
Patients with moderate or severe hypoglycemia (*n*)	7 (27)	6 (30)	5 (22)	5 (23)	13 (28)	10 (23)	12 (25)	11 (27)
Number of moderate or severe hypoglycemia events	10	22	5	20	32	25	15	42
Patients with symptomatic night hypoglycemia (*n*)	13 (50)	4 (15)	5 (22)	4 (19)	17 (37)	9 (20)	18 (37)	8 (19)
Number of nocturnal symptomatic hypoglycemia events	46	16	9	8	62	17	31	25
Patients presenting any type of hypoglycemia (*n*)	16 (61)	14 (70)	15 (68)	12 (57)	30 (65)	27 (62)	31 (64)	26 (60)
Number of any type of hypoglycemia events	113	107	48	111	220	159	157	155

Symptomatic nocturnal hypoglycemia: Symptomatic nocturnal hypoglycemia was defined when hypoglycemia occurred during sleep, after the bedtime insulin dose and before wakening. In this case, hypoglycemia was classified as mild (plasma glucose > 56 mg/dL), moderate (36 mg/dL < plasma glucose < 56 mg/dL) or severe (plasma glucose < 36 mg/dL).

The evaluation of insulin titration was based on the patients' diaries and glycemia levels at every visit. Treatment compliance was evaluated based on the aforementioned information.

RESULTS

Table 3 shows insulin glargine doses in U and U/kg, efficacy parameters, namely FPG and A1C at the end of the study, A1C decrease with respect to baseline value, proportion of patients reaching FPG target (A1C < 7.5% or < 7%), and mean titration time to reach the FPG target in the various groups.

There was no significant difference between the groups in the time required to achieve the target. The safety parameters are shown in Table 4. A unique severe hypoglycemia event (glycaemia < 36 mg/dL) occurred after a prolonged fasting period in a patient randomized according to the DeGold PLUS algorithm. No other severe adverse events occurred.

In a pooled analysis, there was no significant difference when comparing moderate hypoglycemia events in algorithms starting with a 10 U fixed dose with algorithms with BMI variation. However, when we compared patients (n = 46) whose titration increment was 2 U twice a week with patients (n = 43) whose titration varied according to FPG, we observed a clear increase in the number of hypoglycemia events in the second group. We observed 12 hypoglycemia events in the first group, which corresponded to 0.94 events/patient per year, and we observed 42 events in the second group, which corresponded to 2.81 events/patient per year (P < 0.037, Figure 1).

There were no other significant differences in the further comparisons between the algorithms.

DISCUSSION

Titration algorithms are important tools for maximizing the benefits of insulin therapy for metabolic control. Many algorithms have been proposed as guides for achieving metabolic control with basal insulin therapy. These algorithms differ in their initial recommended dos-

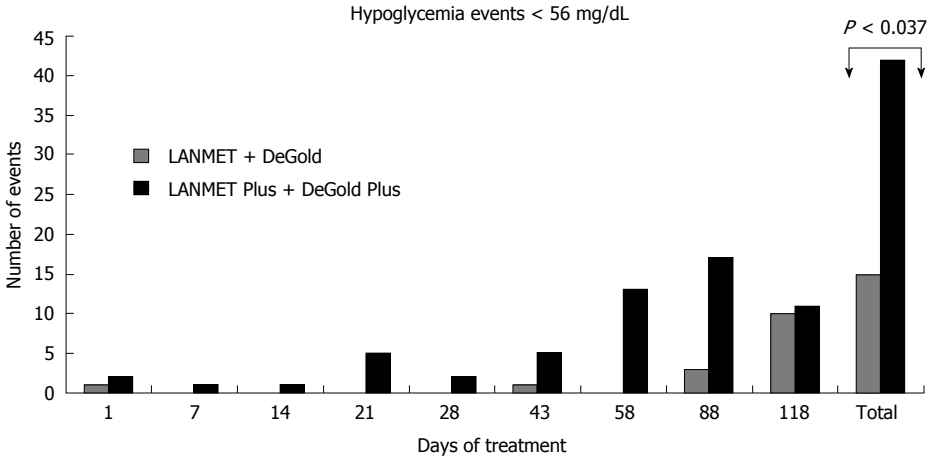

Figure 1 The graphic represents the number of moderate hypoglycemic events occurred throughout the study. The number of hypoglycemia events that occurred in the first 2 wk of treatment was very low. Algorithms that titration increment varied according fasting plasma glucose, had a clear increase in the number of hypoglycemia events.

es, and in the frequency and speed of basal insulin dose adjustments[5,6,11,12]. All were conceived based on the treat-to-target concept, thus becoming comparable in efficiency if correctly used. However, because these algorithms are being used in different populations, it is difficult to compare their safety based on the risk of hypoglycemia because it is unclear whether differences in rates of hypoglycemia are truly due to the algorithm itself or to the patients' varying levels of education. In this study, we evaluated the efficacy and safety of four insulin glargine titration algorithms in a highly homogeneous population to compare the impact of both the initial dose and the titration regimen on hypoglycemia events.

Titration was successfully performed in all groups. The DeGold and DeGold PLUS algorithms used a significantly higher initial insulin doses compared to the other two algorithms, which used a 10 U fixed initial dose. Nevertheless, at the end of the study, the doses were similar in all four groups. The doses were slightly higher (0.67 U/kg) in the DeGold groups, but were comparable to previously reported values (0.69 U/kg) in the LANMET study[11].

As expected, all four algorithms resulted in a decrease in FPG and A1C values, and 85% of all patients actually reached the FPG target and 39% of the patients achieved an A1C < 7% after 18 wk of treatment. This proportion is lower than the 60% reported in the Treat to Target study, where the introduced patient population had lower initial A1C levels (8.6% *vs* 9.5%) and results were reported after 36 wk of treatment. In our case, all groups presented a mean reduction of at least 2% in A1C values.

LANMET is a more conservative algorithm, as it recommends the smallest initial dose and slower titration, as opposed to the DeGold PLUS algorithm, which recommends the initial insulin dose based on BMI and a faster titration protocol. As such, the most important safety outcome to be compared is the frequency of moderate and severe hypoglycemia events, which is a barrier to the acceptance of insulin therapy among clinicians and patients[13,14].

In addition, hypoglycemia is currently acknowledged as risk factor that could lead to cardiovascular events and death[15-22]. Analysis on the incidence of mild, asymptomatic, or total hypoglycemia events showed no significant difference between the groups. However, when comparing the frequency of severe and moderate hypoglycemia events between the two groups on fixed titration and the other two groups using a variable regimen, a significant increase was observed in the latter groups (0.94 events/patient per year *vs* 2.81 events/patient per year, $P < 0.037$).

It has previously been reported that patients typically experience 3 events/patient per year, which is similar to what we observed in the patients who were subject to the titration regimen that varied according to FPG[12]. The frequency of symptomatic hypoglycemia events in the Treat to Target study was higher than in the LANMET trial (4.1 events/patient per year *vs* 13.9 events/patient per year) that used fixed titration, a finding that is in agreement with our observations[12,13].

The performance of the DeGold algorithm was especially notable, and it was recently proposed as an algorithm to guide the introduction of insulin glargine in replacement of OADs for inpatients[8]. We extended its use to outpatients currently being treated with OADs and as a result, after a mean titration period of 22 d, 95% of the individuals reached the FPG target and 69% reached values of A1C < 7%, without increase in any hypoglycemia categories.

Nevertheless, there was no significant difference between the algorithms regarding efficacy parameters, possibly due to a lack of statistical power because of the small sample size.

An increase in the risk of hypoglycemia was associated with the rapid titration algorithms, in comparison to patients receiving higher initial doses. A possible explanation for the observed discrepancy may be the extremely low number of events that occur in the beginning of treatment. Analysis of the distribution of occurrences throughout the study showed that only 14% of all events occurred during the first 4 wk of treatment (data not shown). After this period, the insulin doses in the titration regimens that varied according to FPG were higher, irrespective of the initial dose. Our data suggest that the initial dose is not important for achieving glycemic control, nor was it shown to affect the rates of hypoglycemia

events, as long as titration was performed. However, forced and rapid titration did increase the rates of hypoglycemia events.

In conclusion, there is no increase in the risk of moderate/severe hypoglycemia events when treatment with insulin glargine is initiated on insulin-naïve type 2 diabetes patients using an algorithm where the initial insulin dose is calculated based on BMI, as observed in the DeGold algorithm. However, this risk is increased when a faster titration schedule was used, compared with a fixed 2-U increment twice a week.

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COMMENTS

Background

To start insulin therapy in insulin naïve type 2 diabetes patients, a long-acting basal insulin, such as insulin glargine, is added once a day. The majority of algorithms determine insulin titration according to fasting plasma glucose levels, but the dosage differs at the initial dose, frequency and speed of adjustments.

Research frontiers

It is difficult to compare the different algorithms employed in trials with populations of different socio-economic strata and variable access to educational materials.

Innovations and breakthroughs

Here, authors compared the safety of different titration algorithms in a population that was homogeneous in terms of socio-economic strata and with the same degree of education in diabetes.

Terminology

Insulin algorithm titration: A guideline to modify the insulin dose after starting insulin therapy in a patient.

Peer review

This is an interesting study. The authors tried to compare the safety and efficacy of four insulin glargine algorithms in insulin naive type 2 diabetes patients.

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BRIEF ARTICLE

Rationale, design and baseline patient characteristics of the optimal type 2 diabetes management including benchmarking and standard treatment study in Greece

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Abstract

AIM: To describe baseline data of the optimal type 2 diabetes management including benchmarking and standard treatment (OPTIMISE) study in Greece.

METHODS: "Benchmarking" is the process of receiving feedback comparing one's performance with that of others. The OPTIMISE (NCT00681850) study is a multinational, multicenter study assessing, at a primary care level, whether using "benchmarking" can help to improve the quality of patient care, compared with a set of guideline-based reference values ("non-benchmarking"). In the Greek region, 797 outpatients (457 men, mean age 63.8 years) with type 2 diabetes were enrolled by 84 office-based physicians. Baseline characteristics of this population are presented.

RESULTS: Hypertension was the most prevalent concomitant disorder (77.3%) and coronary heart disease was the most frequent macrovascular complication of diabetes (23.8%). Most patients were overweight or obese (body mass index 29.6 ± 5 kg/m^2), exhibiting mostly abdominal obesity (waist circumference 102.6 ± 13.6 cm). Biguanides were the most prevalent prescribed drugs for the management of diabetes (70.1% of all prescriptions), whereas statins (93.5% of all prescriptions) and angiotensin receptor blockers (55.8% of all prescriptions) were the most prevalent prescribed drugs for hyperlipidemia and hypertension, respectively. Only 37.4% of patients were on aspirin. Despite treatment, pre-defined targets for fasting plasma glucose (< 110 mg/dL), glycated hemoglobin (< 7%), systolic blood pressure (< 130 mmHg and < 125 mmHg for patients with proteinuria) and low density lipoprotein cholesterol levels (< 100 mg/dL and < 70 mg/dL for patients with coronary heart disease) were reached in a relatively small proportion of patients (29%, 53%, 27% and 31%, respectively). In a Greek population with type 2 diabetes, the control of glycemia or concomitant disorders which increase cardiovascular risk remains poor.

CONCLUSION: Despite relevant treatment, there is a poor control of diabetes, hypertension and hyperlipidemia in Greek outpatients with type 2 diabetes.

Key words: Diabetes; Benchmarking; Treatment target; Glycemic control; Dyslipidemia; Blood pressure

Core tip: This is an epidemiological study assessing the prevalence of comorbidities as well as treatment control in a Greek population of patients with type 2 diabetes. "Benchmarking" is the process of receiving feedback and comparing one's performance to that of others. The optimal type 2 diabetes management including benchmarking and standard treatment (OPTIMISE) study is a multinational, multicenter study comparing the efficacy of two follow-up strategies in the manage-

ment of type 2 diabetic outpatients: "benchmarking" *vs* "non-benchmarking". This paper describes the rationale and the design of the OPTIMISE study as well as the baseline characteristics of patients included in the Greek region.

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INTRODUCTION

According to the World Health Organization (WHO), > 180 million people worldwide suffer from diabetes[1]. This number is likely to increase by more than double by the year 2030. In 2005 alone, approximately 1.1 million people died from diabetes-related complications[1]. The WHO projects that without urgent action, deaths due to these complications will increase by > 50% in the next 10 years[1]. Type 2 diabetes, which is closely related to an unhealthy lifestyle and obesity, is associated with increased risk of micro- and macrovascular outcomes, including heart attacks, strokes and amputations of the lower limbs[1]. Furthermore, diabetes complications not only decrease life expectancy, but also markedly reduce the quality of life. These outcomes result in increasing health care costs[2].

This burden can be limited with effective treatment practices[2]. However, a marked variability has been documented in preventive and therapeutic approaches, suggesting that the level of diabetes care currently delivered may not produce the predicted health-related benefits[3]. Gaps between medical care as actually practiced and the recommendations derived from evidence-based research are large and widespread[3]. Approaches improving the quality of patient care include the development of guidelines, flowcharting, data collection and graphical data analysis. More recent innovations are benchmarking and computerized decision support[3].

Benchmarking is the process of comparing one's performance with that of others[4]. This process begins with standardized and comparative measurement. It can go further to understand why there are performance differences between seemingly similar processes[4]. Benchmarking is practical and action-oriented in its analysis; it is not a rigorous research methodology. It is, however, a promising technology that breaks through the isolation that many clinicians report as the underlying cause of variation in clinical practice[4].

The optimal type 2 diabetes management including benchmarking and standard treatment (OPTIMISE, NCT00681850) study was a multinational, multicenter study assessing, at a primary care level, whether using benchmarking can help in improving the quality of patient care as compared with a set of guideline-based reference values. In this paper, baseline data of patients included in the OPTIMISE study in the Greek region are analyzed.

The primary objective of this study was the improvement of the quality of diabetic patient care, particularly the control of glycemia, lipids and blood pressure, with benchmarking over a set of guideline-based reference values (non-benchmarking). In this context, the percentage of patients in the benchmarking group achieving pre-set targets for glycated hemoglobin (HbA1c)[1], low density lipoprotein cholesterol (LDL-C)[1,5] and systolic blood pressure (SBP)[1,6] *vs* non-benchmarking group (control group) after 12 mo of follow-up was assessed.

Secondary objectives were to demonstrate that using benchmarking improves the control of diabetes, lipids and blood pressure (1) by means of the proportion of patients achieving pre-set targets for HbA1c[1], glycemia[1], LDL-C levels[1,5] and SBP[1,6] or (2) by determining the improvement in these parameters after 12 mo of follow-up. Other secondary objectives included (3) the preventive screening for several outcomes: retinopathy, neuropathy, dietary counseling, microalbuminuria, smoking habits, body mass index (BMI) and physical activity and (4) the measurement of physical activity by registering the number of steps and the distance walked per day.

MATERIALS AND METHODS

Study design and population

Type 2 diabetic patients, followed by usual physician treatment, were recruited for observation. Selection criteria were male or female subjects (1) with a minimum age of 18 years; (2) with type 2 diabetes, treated or untreated, insulin dependent or not insulin dependent at the time of first visit; and (3) who signed an informed consent to participate in the study. Diabetes was defined by plasma levels of glucose (PG); fasting PG was ⩾ 126 mg/dL or PG levels 2-h post-load was ⩾ 200 mg/dL. Patients who (1) suffered from type 1 diabetes or gestational diabetes, (2) participated in any other clinical study or (3) were hospitalized during the study period (because it is a primary care study) were excluded from the study.

Investigators recruited for this study were physicians from all over the country who were willing to participate. A selection was based on the availability of sufficient diabetic patients in the physician's practice and the motivation to fulfill the administrative procedures linked to the study. All participating investigators performed their usual monitoring, treatment and counseling of their diabetic patients. Investigators were randomized into two groups. The group that performed the usual monitoring of their diabetic patients by knowing the relative level of diabetic control of their patients compared with the patients of other investigators was defined as the benchmarking group. The other group (non-benchmarking) did not receive any information and behaved as a control group. The proportion of investigators receiving that in-

formation (benchmarking) *vs* the control group was 3 to 1.

Follow-up

All investigators received feedback on the risk factors of their patients. Additionally, in the benchmarking group, physicians anonymously received information on the level of control of cardiovascular risk factors for their patients compared with their colleagues. This possibly resulted in an additional motivational stimulus for investigators and patients to follow therapeutic advice and to improve their risk factors.

The time interval between visits in this study corresponded to the four-times yearly control visits for diabetic patients regarding blood pressure, fasting glycemia, HbA1c, body weight, smoking habits and physical activity, as recommended by the "International Diabetes Federation"[7]. Therefore, according to the study protocol, patients were followed-up in four visits. Baseline assessments were recorded at visit 1, and further data were collected after approximately 4 mo (visit 2), 8 mo (visit 3) and 12 mo (visit 4). The serum lipid profile [total cholesterol (TC), LDL-C, high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels] was also recorded at baseline and at the same time intervals.

Clinical evaluation

At each visit, blood pressure was measured with the patient in the sitting position following at least 5 min of rest with a manometer with a cuff of the recommended dimensions. The mean blood pressure based on three successive readings was recorded. Somatometrics, including body weight, height (only at the first visit) and waist circumference, were also measured during the follow-up. The patient ideally wore light clothing and no shoes during the weight measurement. Weight was given in kilograms, without decimals (to round up as from 0.5 kg). The patient ideally wore no shoes during the height measurement. Height was given in centimeters without decimals (to round up as from 0.5 cm). For the measurement of waist circumference, a measuring tape was placed in a horizontal plane around the abdomen at the level of the iliac crest. Before reading the tape measure, investigators ensured that the tape was snug without compressing the skin and parallel to the floor. The measurement was made at the end of a normal expiration.

Laboratory evaluation

After an 8-h overnight fast, two blood samples (7 mL) were obtained. The following parameters were analyzed at the central lab BARC (Industriepark Zwijnaarde 7b3, B-9052 Ghent, Belgium): (1) HbA1c, (2) fasting PG and (3) the serum lipid profile, including TC, TG, HDL-C and LDL-C levels. At visits 1 and 4, a urine sample of 4 mL was collected for analysis of microalbuminuria.

Pre-defined targets of treatment

Pre-defined targets of treatment were (1) HbA1c < 7% and fasting PG < 110 mg/dL for glycemic control, (2) SBP < 130 mmHg and < 125 mmHg in the case of renal impairment and proteinuria > 1 g/24 h for blood pressure control and (3) LDL-C levels < 100 mg/dL and < 70 mg/dL for very high-risk patients (*i.e.*, those with diabetes and coronary heart disease) for serum lipids control.

Patient classification

Patients were categorized according to fasting PG levels into (1) "normal" if fasting PG was < 110 mg/dL, (2) "borderline" if fasting PG was 110-125 mg/dL and (3) "diabetics" if fasting PG was ⩾ 125 mg/dL. According to HbA1c levels, patients were classified into "good" if HbA1c ⩽ 7% and "too high" if HbA1c > 7%. SBP levels divided the study population into: "good" if < 130 mmHg and "too high" if ⩾ 130 mmHg. According to LDL-C levels, patients were categorized into "good" if LDL-C < 100 mg/dL and "too high" if LDL-C ⩾ 100 mg/dL.

A four-point verbal rating scale was used to assess the following physical activity: (1) no weekly activity; (2) only limited physical activity during most weeks; (3) intense physical activity (activity that gives rise to shortness of breath, tachycardia and sweating) during at least 20 min, once to twice a week; and (4) intense physical activity (activity that gives rise to shortness of breath, tachycardia and sweating) during at least 20 min, three times or more a week.

Statistical analysis

Descriptive statistics (mean, median, number of observations, standard deviation, standard error, 95%CI, minimum and maximum) of all primary and other variables are presented in tables and, if appropriate and interesting, in graphs. This is applicable for the following variables: HbA1c, glycemia, LDL-C, SBP, TG, TC, HDL-C, diastolic blood pressure, waist circumference, smoking habits, microalbuminuria, BMI, physical activity (rating scale), degree of ophthalmic control and degree of dietary advice.

The null hypothesis for the primary objective is that the proportion of patients who reached targets after 12 mo in both groups is equal. The alternative hypothesis is that this proportion is greater in the benchmarking group compared with the control group. This analysis is performed for the following variables: HbA1c, LDL-C and SBP. For secondary objectives, the null hypothesis is that the proportion of patients who reached the target after 12 mo is the same as the proportion of patients who reached the target at baseline. The alternative hypothesis is that this proportion is even greater after 12 mo than at baseline. This analysis is also performed for HbA1c, LDL-C and SBP. Another null hypothesis is that the mean proportion improvement of these variables after 12 mo is equal to zero. The alternative hypothesis is that the mean percentage improvement is different from zero.

RESULTS

The study design and the global baseline results of the OPTIMISE study have been previously reported[8,9]. Ad-

Table 1 History data of the study population in Greece

Variable	Value
Age (yr)	64 ± 11
Male gender	457 (57.3)
Positive family history of diabetes	483 (64.2)
Family history of premature heart disease	213 (28.4)
Time since diagnosis of diabetes (yr)	9.2 ± 8.3
Age at diagnosis of diabetes (yr)	54 ± 11
Smoking status	
Current smokers	194 (24.3)
Ex-smokers	171 (21.4)
Non-smokers	432 (54.2)

Data are expressed as absolute numbers (percentage) or mean ± SD.

Table 3 Baseline clinical characteristics of the study population in Greece (mean ± SD)

Variable	Value
Height (cm)	167 ± 9
Weight (kg)	83 ± 16
Body mass index (kg/m^2)	29.6 ± 5.0
Waist circumference (cm)	103 ± 14
Systolic blood pressure (mmHg)	138 ± 17
Diastolic blood pressure (mmHg)	80 ± 9

ditionally, the benchmarking process has been schematically described in detail above[8].

History data and clinical evaluation

A total of 797 patients were enrolled in this study (n = 570 in the benchmarking group and 227 in the control group) by 84 participating office-based physicians across Greece. History data of the study population are shown in Table 1. Most patients were middle-aged and had a positive family history of diabetes (Table 1). A small predominance of male gender was noted in our population. Patients were middle-aged at the time of diagnosis of diabetes and presented after approximately a 10-year course of diabetes.

Hypertension was a common concomitant disorder in our population, present in approximately 8/10 patients (Table 2). Among macrovascular complications of diabetes, coronary heart disease was the most prevalent, followed by peripheral artery disease and stroke (Table 2). Only two patients have undergone amputation. Retinopathy was the most commonly observed microvascular complication of diabetes, followed by proteinuria (Table 2).

Table 3 shows the main clinical characteristics of the study population. The vast majority of patients were overweight or obese, as reflected by increased BMI. The predominance of visceral obesity was mirrored by abnormally raised measurements of waist circumference. Most patients (*i.e.*, 77%) reported no or light weekly physical activity and the rest (23%) reported "intense physical activity" for 1-2 times per week.

Both systolic and diastolic blood pressure levels were moderately elevated (Table 3). As expected, SBP was greater in patients with proteinuria than in patients without proteinuria (144 ± 19 mmHg *vs* 138 ± 17 mmHg). Only a small proportion of patients (27%) reached the pre-defined target for blood pressure, whereas most patients (72%) did not reach this target (Figure 1).

Table 2 Macrovascular and microvascular complications of diabetes and concomitant diseases of the study population in Greece n (%)

Clinical condition	Value
Hypertension	615 (77.2)
Coronary heart disease	186 (23.8)
Stroke	50 (6.3)
Peripheral artery disease	85 (11.1)
Amputation	2 (0.3)
Proteinuria	38 (5.6)
End-stage renal disease	1 (0.1)
Retinopathy	54 (7.2)

Table 4 Treatment of the study population in Greece

Treatment	n (%)
Antidiabetic	740 (92.9)
Insulin	145 (19.6)
Biguanide (metformin)	519 (70.1)
Sulfonylurea	343 (46.4)
Glitazone	142 (19.2)
Others	104 (14.1)
Lipid-lowering	553 (69.4)
Statin	517 (93.5)
Ezetimibe	53 (9.6)
Fibrate	17 (3.1)
Others	43 (7.8)
Antihypertensive	591 (96.1[1])
ARBs	330 (55.8)
ACEi	202 (34.2)
CCBs	242 (40.9)
Beta-blockers	179 (30.3)
Alpha-blockers	13 (2.2)
Diuretics	220 (37.2)
Others	19 (3.2)
Anti-obesity	40 (5.0)
Aspirin	298 (37.4)

[1]The percentage value refers to patients with hypertension. ARBs: Angiotensin receptor blockers; ACEi: Angiotensin converting enzyme Inhibitors; CCBs: Calcium channel blockers.

Medical treatment

Prescribed medications are shown in Table 4. Biguanides were the most commonly prescribed antidiabetic drugs, followed by sulfonylureas. Approximately one fifth of the patients in our population were treated with insulin. The mean insulin dosage among insulin-treated patients was 48 ± 28 units/d. From all antidiabetic drug combinations, biguanide + sulfonylurea was the most commonly prescribed (20% of all prescriptions).

Almost all patients on lipid lowering therapy were taking statins (Table 4). Simvastatin was used by 34% of the statin-treated patients at a mean dose, atorvastatin by 36% and rosuvastatin by 24% at a mean dose of about 30, 20 and 12 mg/d, respectively. Statins with a mild lipid-lowering potency, including fluvastatin and pravas-

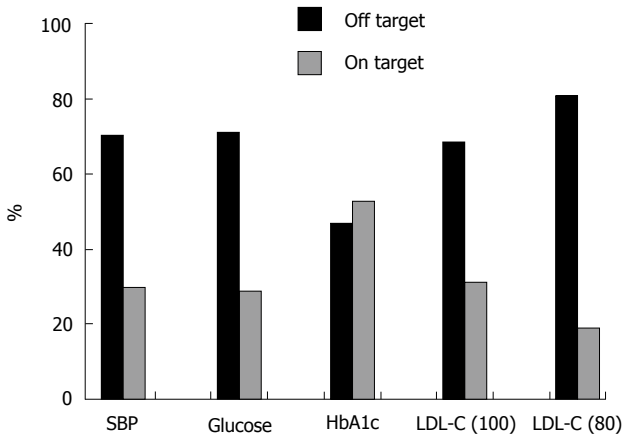

Figure 1 Proportion of patients who did not reach or reached pre-defined targets of treatment. SBP: Systolic blood pressure; HbA1c: Glycosylated hemoglobin; LDL-C: Low density lipoprotein cholesterol.

tatin, were less frequently prescribed. The use of other hypolipidemic drugs was limited in our population. The dose of 100 mg/d was the predominant dose of aspirin, corresponding to 92% of all prescriptions.

Renin-angiotensin-aldosterone system blockade was the most popular antihypertensive strategy, with angiotensin receptor blockers (ARBs) being prescribed in more than half and angiotensin converting enzyme inhibitors (ACEi) in approximately one third of our population (Table 4). ARBs were the most commonly prescribed antihypertensive drug category, followed by calcium channel blockers (CCBs), diuretics, ACEi and beta-blockers (Table 4). From combinations of two antihypertensive drugs, ARBs with diuretics or CCBs were the more prevalent (each representing approximately 5% of all prescriptions), followed by ACEi with the same categories (approximately 3% of all prescriptions for each combination). ARBs, CCBs and diuretics combination were the most frequent among triple combinations (5% of all prescriptions).

Target achievement for laboratory parameters

Table 5 shows the glycemic control and serum lipid profile. Glycemic control was poor, with 71% of all patients being out of the pre-defined target according to fasting PG and 47% according to HbA1c (Figure 1). Interestingly, glycemic control was better when assessed by HbA1c rather than by fasting PG levels.

Only 31% of patients reached the pre-defined target for LDL-C (< 100 and < 70 mg/dL for patients with coronary heart disease). This proportion was greater (*i.e.*, 40%) for the target of LDL-C < 100 mg/dL and lower (19%) for a more aggressive LDL-C target of < 80 mg/dL (Figure 1). Consequently, the LDL-C target was not reached in the vast majority of patients with coronary heart disease (82%).

DISCUSSION

The OPTIMISE study is designed to compare two different strategies in the follow-up of type 2 diabetic

Table 5 Laboratory evaluation of the study population in Greece (mean ± SD)

Variable	Values
Glucose (mg/dL)	138 ± 47
HbA1c (%)	7.2 ± 1.3
LDL-C (mg/dL)	112 ± 35
HDL-C (mg/dL)	50 ± 13
TC (mg/dL)	192 ± 42
TG (mg/dL)	154 ± 85
Albuminuria (mg/g Cr)	66.6 ± 249.2

HbA1c: Glycosylated hemoglobin; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides; Cr: Creatinine.

outpatients regarding the control of diabetes and its concurrent morbidities. Benchmarking is a relatively recent innovation in the quality management sciences, representing a useful tool in the understanding of why there are performance differences between seemingly similar processes[4]. Feedback methods such as benchmarking in which clinicians receive reports of their performance compared with the mean performance of a peer group have been used and studied extensively[3,10]. One underlying theory holds that viewing personal performance within the context of peer performance is a powerful motivator for change[3,11]. In the OPTIMISE study, the hypothesis whether "benchmarking" is superior to a "non-benchmarking" follow-up strategy in the control of diabetes and concurrent morbidities is evaluated.

In the present paper, we discuss baseline characteristics of a relatively large population of type 2 diabetic patients in the Greek region participating in the OPTIMISE study. To the best of our knowledge, this study represents one of the larger diabetes registries in the country.

Type 2 diabetes is becoming an increasingly prevalent morbidity in Greece. In the ATTICA study, the prevalence of diabetes in 3042 subjects who were free of cardiovascular disease was raised from 8% in 2001 to 12.8% in 2006[12]. According to the same study, the age-adjusted five-year incidence of type 2 diabetes was 5.5%[13].

The mean age at diagnosis of diabetes in the OPTIMISE study was 54 years. This finding is in accordance with the "Aegaleo" studies in which the increase in diabetes begins in those > 50 years of age[14]. Interestingly, current data showed clearly that the prevalence is considerably increased after the age of 30 years[15]. Age was found to independently correlate with increased risk for diabetes (OR = 1.07, 95%CI: 1.06-1.08)[15].

In the OPTIMISE study, a mild predominance of the male gender over female was noted. This finding is consistent with epidemiological data from the ATTICA study in which the prevalence of diabetes was higher in men than in women (8% *vs* 6%, respectively)[16]. Likewise, in another analysis, male gender was recognized as an independent predisposing factor for diabetes (OR = 1.43, 95%CI: 1.04-1.95)[15]. The possible explanation for these sex differences may be that men are more susceptible than women to the consequences of indolence and obe-

sity, possibly due to differences in insulin sensitivity and abdominal fat deposition[17].

Most of our diabetic patients had a positive family history of diabetes. It has been shown that Greek subjects with a positive family history of diabetes may have approximately a seven-fold higher risk for diabetes compared with co-responders without a family history of diabetes[15]. Approximately 1/4 of diabetic patients in our population (*i.e.*, 24%) exhibited coronary heart disease, a proportion which is similar to that reported for the prevalence of diabetes among Greek patients who had suffered a myocardial infarction (*i.e.*, 25%)[18]. Coronary heart disease represented the most prevalent disorder among all macrovascular complications of diabetes, with the rates for other forms of cardiovascular disease being relatively low.

The prevalence of hypertension was high among our subjects. Hypertension was considered as an independent contributing factor for diabetes in Greek adult subjects with self-reported diabetes (OR = 2.19, 95%CI: 1.60-2.99)[15]. The prevalence of hypertension in our population was greater compared with that recorded in an urban Greek population of self-reported diabetes (77% *vs* 51%, respectively)[15]. The great prevalence of hypertension among Greek subjects with metabolic syndrome (*i.e.*, 71%)[19], which represents a pre-diabetic condition, may account for high rates of hypertension in type 2 diabetic patients.

According to BMI values, approximately all patients were overweight or obese with increased measurements of waist circumference. Being overweight and obese was associated with a two-fold increase in the risk for diabetes in a Greek population[15]. Abdominal obesity, which is present in 82% of patients with metabolic syndrome in Greece[19], may play a major role in the pathogenesis of type 2 diabetes by promoting insulin resistance[20]. Physical inactivity was another important finding of this study. The proportion of our diabetic patients who reported physical inactivity was greater than that recorded in the ATTICA study (77.41% *vs* approximately 50%, respectively)[19]. This unhealthy lifestyle pattern could be related to the development of obesity and diabetes.

The most important finding of this study lies in the low rates of patients who reached pre-defined targets of treatment for SBP, glycemia and LDL-C levels. Approximately 72% of patients were off target regarding SBP. This rate is in accordance with the Didima study, which shows that only 27% of treated hypertensive subjects reached treatment targets for arterial blood pressure in a rural Greek area[21]. In the EUROASPIRE Ⅱ study, 50% of patients with coronary heart disease in 15 European countries (including Greece) had raised blood pressure levels[22]. Similar were the results of a Greek trial performed in patients with coronary heart disease of whom only 50% had desirable blood pressure levels[18].

Suboptimal control was noted for LDL-C levels. Seven out of 10 patients did not reach the pre-defined target of LDL-C levels < 100 mg/dL and < 70 mg/dL for diabetic patients with coronary heart disease. This rate was even lower for a more promising target of < 80 mg/dL. Interestingly, this was evident despite high rates of patients who were treated with statins (*i.e.*, 65% of the total study population or 94% of those receiving lipid lowering medications), particularly the most potent ones. Nevertheless, few patients were treated with drugs that could offer further LDL-C lowering, including ezetimibe. Lipid-lowering drug combinations, which are currently underused, could contribute to a greater percentage of patients reaching the targets for LDL-C levels. In the EUROASPIRE Ⅱ study, 58% of patients with coronary heart disease had elevated TC levels[22]. In Greece, the OLYMPIC study showed that only 26% of 2660 adults with dyslipidemia, who had been receiving lipid-lowering treatment for at least 3 mo (of whom 36% had diabetes), achieved the NCEP-ATPIII targets for LDL-C levels[23]. A greater proportion (*i.e.*, 49%) of patients achieving the 2004-updated NCEP ATPIII targets was reported in the CEPHEUS (Centralized Pan-European survey on the undertreatment of hypercholesterolemia in patients using lipid lowering drugs). This study was performed in 1321 Greek patients who were on lipid lowering treatment for at least 3 mo were stable for at least 6 wk. Interestingly, 25% of these patients had diabetes[23].

In the OPTIMISE study, 34% of the statin-treated patients were on simvastatin, most of them at low doses (49% of them 20 mg/d and 9% 10 mg/d). If these patients were switched to a more potent statin (either atorvastatin or rosuvastatin), they might have reached the targets for LDL-C. Moreover, > 36% of patients on atorvastatin were using low-to-moderate doses (38% of them 10 mg/d and 44% 20 mg/d). It is possible that these patients would reach their targets if titrated to a higher atorvastatin dose or switched to a more potent statin, such as rosuvastatin. Finally, 24% of patients were treated with rosuvastatin (76% of patients used 5-10 mg/d and only 22% 20 mg/d). A higher rosuvastatin dose could potentially offer a higher proportion of patients achieving LDL-C goals. According to international recommendations, statin treatment should be optimized and if the target is not reached, then a second agent should be added. Nevertheless, it appears that statin treatment was far from optimal in the OPTIMISE population. An optimization of statin dose or switching to a more potent statin could help more patients reach the target. If the target is not reached, then the addition of a second agent could be useful.

Poor glycemic control was also noted in our population; only 30% according to fasting PG levels and 50% according to HbA1c levels. The results of the EUROASPIRE Ⅱ study were similar among diabetic patients with coronary heart disease, with more than 70% being out of target for PG levels[21]. In a Greek population of 819 diabetic patients with coronary heart disease, only half of the patients exerted HbA1c levels < 7.5%[18]. Although insulin is considered as a first-line treatment choice for the management of type 2 diabetic patients, only one fifth of patients in the OPTIMISE study were treated with insulin. This could have attributed to low

rates of glycemic control.

The OPTIMISE study was designed to compare the efficacy of "benchmarking" compared with "non-benchmarking" in the control of type 2 diabetes in an outpatient basis. In Greek participants of this study, poor control of diabetes, hypertension and hyperlipidemia were noted at baseline despite treatment.

COMMENTS

Background

The optimal type 2 diabetes management including benchmarking and standard treatment (OPTIMISE, NCT00681850) study was a multinational, multicenter study assessing, at a primary care level, whether using benchmarking can help more in improving the quality of patient care as compared with a set of guideline-based reference values.

Research frontiers

Benchmarking is practical and action-oriented in its analysis; it is not a rigorous research methodology. It is, however, a promising technology that breaks through the isolation that many clinicians report as the underlying cause of variation in clinical practice.

Innovations and breakthroughs

The OPTIMISE study was designed to compare the efficacy of "benchmarking" compared with "non-benchmarking" in the control of type 2 diabetes on an outpatient basis.

Applications

Despite relevant treatment, there is a poor control of diabetes, hypertension and hyperlipidemia in Greek outpatients with type 2 diabetes.

Peer review

The manuscript is well-written and its aim to confirm the importance of benchmarking to improve diabetes care in clinical setting is of interest.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 200201 03498. 2002 Aug 1

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