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Editorial Board Member of *World Journal of Diabetes*, Sze M Ng, MBBS, FHEA, FRCPCH, SFFMLM, MSc, LLM, MBA, PhD, Associate Professor, University of Liverpool, Consultant Paediatric Endocrinologist, Southport & Ormskirk NHS, Ormskirk L39 2AZ, United Kingdom. may.ng@nhs.net

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EXPERT RECOMMENDATIONS

Expert opinion on the preoperative medical optimization of adults with diabetes undergoing metabolic surgery

Saptarshi Bhattacharya, Sanjay Kalra, Nitin Kapoor, Rajiv Singla, Deep Dutta, Sameer Aggarwal, Deepak Khandelwal, Vineet Surana, Atul Dhingra, Viny Kantroo, Sachin Chittawar, Nilakshi Deka, Vivek Bindal, Puja Dutta

ORCID number: Saptarshi Bhattacharya 0000-0002-8458-9371; Sanjay Kalra 0000-0003-1308-121X; Nitin Kapoor 0000-0002-9520-2072; Rajiv Singla 0000-0002-9674-6872; Deep Dutta 0000-0003-4915-8805; Sameer Aggarwal 0000-0003-4568-8668; Deepak Khandelwal 0000-0002-8185-8206; Vineet Surana 0000-0002-6630-2672; Atul Dhingra 0000-0003-3387-9157; Viny Kantroo 0000-0001-7477-6037; Sachin Chittawar 0000-0003-3217-717X; Nilakshi Deka 0000-0002-7575-7329; Vivek Bindal 0000-0003-1003-9446; Puja Dutta 0000-0002-6551-7137.

Author contributions: Bhattacharya S, Kalra S, and Singla R conceived the study; Kapoor N, Kandelwal D, Dutta D, and Surana V conducted the literature search; All authors attended a meeting held at the sidelines of the Annual Society for Promotion of Education in Endocrinology and Diabetes Conference in February 2020, and multiple virtual meetings were attended by the authors to reach a consensus on controversial topics; Bhattacharya S, Chittawar S, Dutta P, and Deka N prepared the initial draft of the manuscript; Aggarwal S, Dhingra A, Bindal V, and Kapoor N revised the manuscript; All authors have read and approved the final manuscript.

Saptarshi Bhattacharya, Endocrinology, Max Superspeciality Hospital, New Delhi 110092, India

Sanjay Kalra, Endocrinology, Bharti Hospital, Karnal 132001, Haryana, India

Nitin Kapoor, Endocrinology, Christian Medical College, Vellore 632004, Tamil Nadu, India

Rajiv Singla, Endocrinology, Kalpavriksh Super Speciality Center, New Delhi 110075, India

Deep Dutta, Endocrinology, CEDAR Superspecialty Clinic, New Delhi 110075, India

Sameer Aggarwal, Endocrinology, Apex Plus Superspeciality Hospital, Rohtak 124001, Haryana, India

Deepak Khandelwal, Endocrinology, Maharaja Agrasen Hospital, New Delhi 110026, India

Vineet Surana, Endocrinology, Manipal Hospitals, New Delhi 110075, India

Atul Dhingra, Endocrinology, Gangaram Bansal Super Speciality Hospital, Sri Ganganagar 335001, Rajasthan, India

Viny Kantroo, Respiratory Medicine & Critical Care, Indraprastha Apollo Hospitals, Sarita Vihar, New Delhi 110076, India

Sachin Chittawar, Endocrinology, Gandhi Medical College, Bhopal 462001, Madhya Pardesh, India

Nilakshi Deka, Endocrinology, Apollo Hospitals, Guwahati 781005, Assam, India

Vivek Bindal, Minimal Access, Metabolic and Bariatric surgery, Max Superspeciality Hospital, Patparganj, New Delhi 110092, India

Puja Dutta, Nutrition, Max Superspeciality Hospital, Patparganj, New Delhi 110092, India

Corresponding author: Saptarshi Bhattacharya, FACE, MD, Doctor, Endocrinology, Max Superspeciality Hospital, Patparganj, 108A I P Extension, New Delhi 110092, India. saptarshi5@yahoo.com

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Abstract

Diabetes mellitus (DM) and obesity are interrelated in a complex manner, and their coexistence predisposes patients to a plethora of medical problems. Metabolic surgery has evolved as a promising therapeutic option for both conditions. It is recommended that patients, particularly those of Asian origin, maintain a lower body mass index threshold in the presence of uncontrolled DM. However, several comorbidities often accompany these chronic diseases and need to be addressed for successful surgical outcome. Laparoscopic Roux-en-Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (LSG) are the most commonly used bariatric procedures worldwide. The bariatric benefits of RYGB and LSG are similar, but emerging evidence indicates that RYGB is more effective than LSG in improving glycemic control and induces higher rates of long-term DM remission. Several scoring systems have been formulated that are utilized to predict the chances of remission. A glycemic target of glycated hemoglobin < 7% is a reasonable goal before surgery. Cardiovascular, pulmonary, gastrointestinal, hepatic, renal, endocrine, nutritional, and psychological optimization of surgical candidates improves perioperative and long-term outcomes. Various guidelines for preoperative care of individuals with obesity have been formulated, but very few specifically focus on the concerns arising from the presence of concomitant DM. It is hoped that this statement will lead to the standardization of presurgical management of individuals with DM undergoing metabolic surgery.

Key Words: Diabetes; Obesity; Metabolic surgery; Bariatric surgery; Remission of diabetes

Core Tip: The ambit of metabolic surgery for diabetes has increased. Individuals with inadequate glycemic control can be considered for surgery if less severely obese, and even more so if they are of Asian origin. However, both diabetes and obesity are associated with multiple comorbidities that require optimization before surgery. There are several clinical guidelines for the preoperative management of individuals with obesity; however, specific suggestions addressing these concerns in persons with diabetes have not been recommended. It is important to achieve optimal glycemic control and diagnose and manage cardiovascular, pulmonary, gastrointestinal, and renal complications before surgery. Nutritional assessment, psychological evaluation, and ruling out specific endocrine disorders are other essential adjuncts. These guidelines will help to standardize the management of preoperative comorbidities and improve postoperative outcomes in individuals with diabetes who opt for metabolic surgery.

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INTRODUCTION

The twin epidemics of diabetes mellitus (DM) and obesity have enormous medical as well as financial implications. Both are chronic and usually life-long conditions with very few definitive therapeutic choices that alter their natural course[1]. Metabolic surgery, which was commonly designated earlier as bariatric surgery, has emerged over the last three decades as a potentially disease-modifying option for both these disorders. The terms "bariatric surgery" and "metabolic surgery" have often been used interchangeably. Most societies now endorse the term "metabolic surgery" as weight-dependent and weight-independent benefits of these procedures are gradually

being recognized[2,3].

DM and uncontrolled hyperglycemia have emerged as important determinants of the need for metabolic surgery in individuals with obesity. DM is associated with multiple comorbidities that demand individualized attention around the bariatric procedure. Although there are several guidelines that address the preoperative concerns before metabolic surgery, none of them specifically focus on the issues arising in DM. This statement provides recommendations on preoperative medical management for individuals with DM who plan to undergo metabolic surgery.

DEVELOPMENT OF GUIDELINES AND GRADING OF SCIENTIFIC EVIDENCE

The expert panel met at the Society for Promotion of Education in Endocrinology and Diabetes Conference (SPEEDCON) 2020, the third annual conference of SPEED, held on 1-2, February 2020, at Gurugram, Haryana, India. The authors searched the medical literature in the PubMed related to bariatric or metabolic surgery for patients with obesity and DM. Search terms included "bariatric surgery" or "metabolic surgery", and "diabetes mellitus" in combination with the terms related to the sections that were planned to be addressed in the statement. The latter search words included "indications"; "type of surgery" and all of the common types of metabolic surgery commonly performed *e.g.*, "laparoscopic Roux-en-Y gastric bypass," "laparoscopic sleeve gastrectomy," *etc.*; "remission" and "predictors of remission," "glycemic status," "glycemic control," "glycemic management" with and without the term "perioperative"; "cardiovascular disease"; "hypertension," "blood pressure," "dyslipidemia" and "lipid profile"; "pulmonary," "respiratory," "tobacco," "smoking," "pulmonary function test," "obstructive sleep apnea," "obesity hypoventilation syndrome" and "venous thromboembolism"; "gastrointestinal," "upper gastrointestinal endoscopy," "gastroesophageal reflux disease," and "*Helicobacter pylori*"; "hepatic," "liver," "non-alcoholic fatty liver disease," and "nonalcoholic steatohepatitis"; "renal," "kidney," "creatinine," "albumin-creatinine ratio," "electrolytes," "sodium," "potassium," and "uric acid"; "nutrition," "iron," "vitamin B12," "folic acid," "anemia," "vitamin D," "vitamin A," "vitamin K," "vitamin E," "copper," "zinc," and "selenium"; "hypothyroidism," "thyroid function test," "Cushing's syndrome," "polycystic ovary syndrome," "pregnancy," "hypogonadism," "monogenic obesity" and "syndromic obesity"; "psychological" and "behavioral"; and "preoperative weight loss," "low calorie diet" and "very low calorie diet."

The authors followed the system developed by the American Diabetes Association (ADA) to grade the quality of scientific evidence supporting the recommendations (Table 1)[4]. The recommendations were allotted grades of A, B, or C based on the nature of the available evidence. Expert opinion E was ascribed to recommendations that lack evidence from clinical trials, where clinical trials may not be feasible, or the available literature is inconclusive. However, it is imperative to understand that although scientific evidence and recommendations can be crucial guiding principles, the management of every patient should ultimately be individualized for each particular case[5,6].

PROBLEM STATEMENT: PREVALENCE OF OBESITY AND DIABETES

Obesity is a common problem that has grown into a global health and economic crisis. The World Health Organization (WHO) defines overweight and obesity as 'abnormal or excessive fat accumulation that presents a health risk'[7]. According to the WHO 2016 global estimates, 39% of adults were overweight, and 13% were obese[8]. The Center for Disease Control and Prevention 2017 data suggested that 42.4% of adults in the United States of America were obese, while 9.2% were severely obese[9].

The increase in the prevalence of obesity has been accompanied by a parallel upsurge in cases of DM[10]. The International Diabetes Federation declared the current global prevalence rate of DM to be 9.3% (463 million), and predicts that it will go up to 10.2% (578 million) by 2030[11]. Both DM and obesity share a common pathogenesis, and the term "diabesity" has often been used when the two conditions coexist[12]. Obesity is recognized as a risk factor for the development of type 2 DM (T2DM)[13,14]. The coexistence of DM and obesity adversely affects the outcome of each condition and exerts an unfavorable cardiovascular impact[15].

Table 1 Evidence grading system for recommendations

Level of evidence	Description
A	Clear evidence from well-conducted, generalizable randomized controlled trials that are adequately powered, including: Evidence from a well-conducted multicenter trial; Evidence from a meta-analysis that incorporated quality ratings in the analysis. Compelling nonexperimental evidence, *i.e.* "all or none" rule developed by the Centre for Evidence-Based Medicine at the University of Oxford. Supportive evidence from well-conducted randomized controlled trials that are adequately powered, including: Evidence from a well-conducted trial at one or more institutions; Evidence from a meta-analysis that incorporated quality ratings in the analysis
B	Supportive evidence from well-conducted cohort studies: Evidence from a well-conducted prospective cohort study or registry; Evidence from a well-conducted meta-analysis of cohort studies. Supportive evidence from a well-conducted case-control study
C	Supportive evidence from poorly controlled or uncontrolled studies: Evidence from randomized clinical trials with one or more major or three or more minor methodological flaws that could invalidate the results. Evidence from observational studies with high potential for bias (such as case series with comparison with historical controls); Evidence from case series or case reports. Conflicting evidence with the weight of evidence supporting the recommendation
E	Expert consensus or clinical experience

CLASSIFICATION OF OBESITY AND DIABETES

The WHO has classified obesity based on body mass index (BMI). Obesity is conventionally defined as BMI ≥ 30 kg/m^2, while BMI between 25.0 and 29.9 kg/m^2 is defined as overweight[7]. Asians have higher body fat percentage at lower values of BMI[16,17]; thus, more stringent criteria have been used to define obesity in the Asian population[18,19]. Table 2 depicts the classification system used to define obesity internationally and for Asia.

The ADA and WHO criteria are the established methods for diagnosing DM[20,21]. The WHO does not support the use of glycated hemoglobin (HbA1c) for diagnosing DM[21]. The ADA classifies DM into four categories: Type 1 DM (T1DM), T2DM, gestational DM, and other specific types of DM[20]. T2DM is closely inter-related to obesity and comprises the predominant subtype of DM encountered in patients undergoing metabolic surgery.

INDICATIONS FOR METABOLIC SURGERY IN DIABETES

Recommendation 1

Metabolic surgery is recommended as a therapeutic option in T2DM if the BMI is ≥ 40 kg/m^2 (≥ 37.5 kg/m^2 for Asians) irrespective of glycemic status (A). Surgery is also recommended as a treatment modality in T2DM with BMI between 35 to 39.9 kg/m^2 (32.5 to 37.4 kg/m^2 for Asians) if adequate glycemic control cannot be achieved despite standard management (B).

Recommendation 2

Metabolic surgery should be considered as a therapeutic option in T2DM with BMI between 30 to 34.9 kg/m^2 (27.5 to 32.4 kg/m^2 for Asians) if glycemic control is suboptimal despite standard management (B). However, the committee recognizes that there is limited evidence to support the long-term efficacy of metabolic surgery in Asians with T2DM and BMI < 30 kg/m^2, and scrutiny of risk *vs* benefit should be undertaken before performing the procedure in patients with lower BMI (E).

Recommendation 3

The associated conditions that might favor a surgical approach in T2DM with obesity are poorly controlled hypertension, non-alcoholic fatty liver disease (NAFLD), obstructive sleep apnea (OSA), obesity hypoventilation syndrome (OHS), osteoarthritis of the knee or hip, urinary stress incontinence, polycystic ovary syndrome (PCOS), gastro-esophageal reflux disease (GERD), idiopathic intracranial hypertension, severe venous stasis disease, obesity-related limited mobility and poor quality of life (E).

Discussion

The 2nd Diabetes Surgery Summit (DSS-II) defined the eligibility for metabolic surgery in T2DM with obesity, depending on the adequacy of glycemic control in conjunction with BMI[22]. Our committee broadly endorses the criteria for metabolic surgery as

Table 2 Obesity classification system for adults: International and Asian

Category	WHO International classification BMI (kg/m²)	Asian classification BMI (kg/m²)
Underweight	< 18.5	< 18.5
Normal weight	18.5-24.9	18.5-22.9
Overweight	25.0-29.9	23-24.9
Obesity class I	30.0-34.9	25-29.9
Obesity class II	35.0-39.9	30-34.9
Obesity class III	≥ 40	≥ 35

BMI: Body mass index.

specified in the DSS-II recommendations. The indication for metabolic surgery along with level of existing evidence is summarized in Table 3.

BMI (≥ 35 kg/m²)

Long-term efficacy of metabolic surgery in improving the outcome of T2DM with BMI ≥ 35 kg/m² has been clearly demonstrated. Meta-analyses has shown that macrovascular and microvascular outcomes, and mortality are significantly better after metabolic surgery than medical therapy[23,24]. The meta-analysis by Yan *et al*[25] specifically looked into outcomes of studies with more than 5 years of follow-up. Surgery resulted in a lower incidence of macrovascular complications (relative risk [RR] = 0.43), all-cause mortality (hazard ratio [HR] = 0.65), lower weight, and better glycemic control compared to medical management. Long-term observational data from Swedish Obese Subjects registry also demonstrate the benefit of surgery in terms of DM remission (median follow-up 10 years) as well as macrovascular and microvascular complications (median follow-up 17.6 years for surgery and 18.1 years for controls) over medical therapy[26].

BMI (30-34.9 kg/m²)

Evidence also support the beneficial role of metabolic surgery in individuals with DM and BMI < 35 kg/m²[27,28]. In the meta-analysis by Müller-Stich *et al*[29] surgery resulted in a higher T2DM remission rate (odds ratio [OR] = 14.1), better rates of glycemic control (OR = 8.0) and lower HbA1c in individuals with DM and BMI < 35 kg/m² compared to standard medical management. Long-duration randomized controlled trials (RCTs) can help further substantiate this recommendation.

BMI (< 30 kg/m²)

In a meta-analysis of 12 studies done by Ji *et al*[30], 697 Asian subjects with DM and BMI < 30 kg/m² were analyzed at 6, 12, and 24 mo after metabolic surgery. After 1 year of surgery, BMI and waist circumference decreased by 2.88 kg/m² and 12.92 cm, respectively. Improvement in glycemic and lipid parameters was also observed at all three timepoints. Another meta-analysis of 26 studies assessed the remission of DM in subjects with a BMI < 30 kg/m². The follow-up duration ranged from 6 to 42.1 mo, with half of the studies having data for 12 mo only. The mixed-effect meta-analysis model estimated an overall DM remission of 43% along with an HbA1c reduction of 2.08%[31]. However, long-term outcome data to support the application of metabolic surgery in Asians with DM and BMI < 30 kg/m² is necessary before its routine clinical application.

Comorbidities

Metabolic surgery in subjects with obesity and DM demonstrated a favorable effect on hypertension[29,32]. The recent meta-analysis by Yan *et al*[25] however failed to show benefit in blood pressure after a minimum follow-up of 5 years. Various other obesity-related comorbidities improved after bariatric procedures, but specific evidence in subgroups with DM is lacking. The clinical practice guidelines by the American Association of Clinical Endocrinologists (AACE)/American College of Endocrinology, The Obesity Society (TOS), the American Society for Metabolic & Bariatric Surgery (ASMBS), the Obesity Medicine Association (OMA), and the American Society of Anesthesiologists (ASA) in 2019 suggested that bariatric procedures can be considered

Table 3 Indications for metabolic surgery in obesity along with presence of diabetes

Condition	Glycemic status	Recommendation for metabolic surgery	Evidence category
Diabetes and BMI ≥ 40 kg/m^2 (≥ 37.5 kg/m^2 for Asians)	Any	Strong recommendation	A
Diabetes and BMI 35-39.9 kg/m^2 (32.5-37.4 kg/m^2 for Asians)	Uncontrolled despite optimal treatment	Moderate recommendation	B
Diabetes and BMI 30-34.9 kg/m^2 (27.5-32.4 kg/m^2 for Asians)	Uncontrolled despite optimal treatment	Weak recommendation	C, E
Diabetes and obesity (BMI – not defined) with comorbidities: Poorly controlled hypertension; Non-alcoholic fatty liver disease; Obstructive sleep apnea; Obesity hypoventilation syndrome; Osteoarthritis of the knee or hip; Urinary stress incontinence; Polycystic ovary syndrome; Gastro-esophageal reflux disease; Idiopathic intracranial hypertension; Severe venous stasis disease; Obesity-related limited mobility; Obesity-related poor quality of life	Any	Weak recommendation	E

BMI: Body mass index.

in obese subjects with BMI > 35 kg/m^2 in the presence of comorbidities such as NAFLD, OSA, osteoarthritis of the knee or hip, and urinary stress incontinence. The guideline also recognized beneficial but weak evidence supporting the role of surgery for the amelioration of OHS, idiopathic intracranial hypertension, GERD, severe venous stasis disease, obesity-related limited mobility, and impaired quality of life [33]. Weak evidence also exists regarding improvement in fertility, menstrual irregularity, and hirsutism in women with PCOS after bariatric procedures[34]. Our expert committee advocates that metabolic surgery should be considered as a therapeutic option in obesity and DM, especially if associated with comorbidities that improve after bariatric procedures. However, the committee acknowledges that evidence in favor of such a recommendation is very weak and should be substantiated by further research.

CHOICE OF THE TYPE OF METABOLIC SURGERY IN DIABETES

Recommendation 4

Laparoscopic Roux-en-Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (LSG) are the two most preferred bariatric procedures worldwide. RYGB and LSG result in equivalent long-term weight loss, with RYGB producing better glycemic control than LSG on prolonged follow-up and can be the preferred bariatric procedure in presence of DM (B). Other factors that might guide the choice of type of surgery are the risk of nutritional deficiencies resulting from malabsorption after RYGB and the possibility of GERD development after LSG. Institutional expertise can also guide the decision regarding the choice of the type of surgery (E).

Recommendation 5

Laparoscopic adjustable gastric banding (LAGB) is an effective procedure in inducing weight loss. The risk of complications related to the gastric band and possible need for revision surgery in the future should be taken into consideration before undertaking LAGB (B).

Recommendation 6

Biliopancreatic diversion (BPD) or BPD with duodenal switch (BPD-DS) is the most effective procedure in causing weight loss and remission of DM but has the maximum risk of immediate postoperative and long-term complications and should only be reserved for those having extremely high BMI (> 60 kg/m^2) (B).

Discussion

The four standard bariatric procedures include RYGB, LSG, LAGB, BPD, or BPD-DS. There are many other variations of these procedures. Several endoscopic techniques have also emerged as means to induce weight loss in recent years. A systemic review reported the weighted means of the percentage of excess weight loss (%EWL) at 10

years or more after BPD ± DS, RYGB, LSG, and LAGB to be 74.1%, 55.4%, 57%, and 45.9%, respectively[35].

Long-term outcome in obesity studies

A recently published meta-analysis of 18 studies (9 RCTs and 9 non-randomized interventions) comprising 2917 participants demonstrated that both RYGB and LSG had similar efficacy in causing weight reduction and remission of DM. The postoperative complication and reoperation rates were less with LSG than RYGB. However, improvement in dyslipidemia, hypertension, and GERD was better with RYGB compared to LSG[36]. Another meta-analysis of 28 studies (7 RCTs, 6 prospective observational studies, and 15 retrospective observational studies) including 9038 subjects with obesity, revealed higher remission rates of T2DM with RYGB after 3 years in comparison to the LSG group. Five-year follow-up data showed that RYGB was superior to LSG in terms of weight loss, T2DM remission, and improvement in hypertension and dyslipidemia (low-density lipoprotein [LDL])[37].

Long-term outcome in subjects with diabetes

In the meta-analysis by Madadi *et al*[38], T2DM remission rates in the LSG group were significantly (OR = 0.71, P = 0.003) less than that of the RYGB group, though the difference lost significance after 1 year. However, more DM remission was achieved with LSG compared to LAGB (OR = 2.17, P = 0.001) after 1 year[38]. Other meta-analyses have also demonstrated the superiority of RYGB over LSG in improving weight loss, and short and mid-term glycemic and lipid parameters in patients with and without T2DM[39,40]. Another meta-analysis revealed that DM resolution was highest after BPD (89%), followed by RYGB (77%), LAGB (62%), and LSG (60%)[41]. In STAMPEDE, one of the landmark trial in metabolic surgery, RYGB fared better than LSG at 5 years in achieving better glycemic control. Besides, the RYGB group required less medicine for glycemic control as compared to the LSG group[42]. Meta-analyses also revealed that immediate complication rates were higher after RYGB, and the risk of repeat surgery was higher after LAGB[35,43]. The postoperative and long-term complications were highest after BPD/BPD-DS, and the DSS-II statement suggested that these procedures should be reserved for extreme cases of obesity (BMI > 60 kg/m^2)[22,44]. A comparison of the outcomes of RYGB and LSG in patients with DM and obesity is summarized in Table 4.

PREDICTORS FOR REMISSION OF DIABETES

Recommendation 7

Remission of DM can be defined as HbA1c < 6.5% and fasting plasma glucose (FPG) < 126 mg/dL (7 mmol/L) along with complete discontinuation of glucose-lowering therapy that persists for at least 6 mo (E).

Recommendation 8

We suggest that partial remission of DM can be defined as HbA1c < 5.7% and FPG < 100 mg/dL (5.6 mmol/L) persisting for at least 6 mo, when metformin is continued (E).

Recommendation 9

Preoperative fasting C-peptide level, younger age, shorter duration of DM, preoperative glycemic status, and pre-surgery requirement for insulin act as indices of pancreatic beta-cell reserve, and correlate with the chance of remission. BMI, visceral fat area (VFA), and waist circumference act as indicators of potential for reducing insulin resistance, and can also predict remission (A). Prediction models like DiaRem score, ABCD, and Individualized Metabolic Surgery (IMS) scores are validated methods to assess remission probability (B).

Discussion

Definition of remission: The most commonly applied criteria for defining DM remission was proposed by Buse *et al*[45]. Partial remission was defined as, HbA1c < 6.5%, and FPG between 100–125 mg/dL (5.6–6.9 mmol/L) lasting for 1 year or more after the procedure, in the absence of pharmacologic therapy. Complete remission was defined as HbA1c in normoglycemic range (< 5.7%) and FPG < 100 mg/dL (5.6 mmol/L) for at least 1 year. Prolonged remission or "cure" was considered as a

Table 4 Comparison between laparoscopic Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy, the two most commonly performed bariatric procedures, in patients with diabetes and obesity

	RYGB	LSG	Comments
Type of procedure	Combined malabsorptive and restrictive	Restrictive	
Effect on weight loss	+++	+++	Most studies demonstrate comparable weight loss, with slight superiority of RYGB shown in some reports
Remission of diabetes	+++	++	RYGB superior to LSG
Short term glycemic improvement	+++	++	RYGB superior to LSG
Long term glycemic improvement	+++	++	RYGB superior to LSG
Improvement in hypertension	++	+	RYGB superior to LSG
Improvement in dyslipidemia	++	+	RYGB superior to LSG
Improvement in gastroesophageal reflux disease	++	+	RYGB superior to LSG
Postoperative complications	+	+/-	Postoperative complication and reoperation rates less with LSG than RYGB
Long-term nutritional deficiencies	++	+	LSG safer that RYGB

LSG: Laparoscopic sleeve gastrectomy; RYGB: Laparoscopic Roux-en-Y gastric bypass.

complete remission lasting for 5 years or more. The stringent criteria proposed in the statement have the drawback of using different thresholds for diagnosis of DM and complete remission. Besides, many individuals who receive metformin for prophylactic purpose will not satisfy this criterion despite having HbA1c in the normoglycemic range. A definition of DM remission has also been proposed by the Association of British Clinical Diabetologists (ABCD) and the Primary Care Diabetes Society[46]. DM remission has also been defined by Kalra *et al*[47] and our panel approves the definition suggested by them. Our committee also proposes that partial remission can be defined as patients receiving metformin, and having HbA1c < 5.7% and FPG < 100 mg/dL (5.6 mmol/L) for a minimum duration of 6 mo. The HbA1c lowering effect of metformin varies between 1.12 to 0.6%. We consider that a cut-off HbA1c < 5.7% along with metformin is a reasonable approximation to the Hba1c value of 6.5% without the drug[48]. Though there is absence of outcome data in candidates receiving prophylactic metformin post-surgery, defining such a group will enable researchers to assess the usefulness of the strategy.

Predictors for remission

The rates of DM resolution after different types of metabolic surgery have already been discussed in the preceding section. DM remission results from the interplay of pancreatic beta-cell reserve and the potential for the decrement in insulin resistance [49]. The indicators of beta-cell reserve that correlate with remission include short DM duration, absence of insulin use, better glycemic control, higher serum C-peptide levels, lower age and lesser number of DM medicines[50-55]. The surrogate indices of insulin resistance with predictive value are high baseline BMI, wider waist circumference, hepatic steatosis, VFA, and inflammatory markers such as high serum C-reactive protein (CRP) and osteopontin[50,52,53,56-60].

In the meta-analysis by Wang *et al*[61], younger age, short DM duration, better glycemic control (lower HbA1c level), and absence of insulin use, correlated with remission. Asian patients were more likely to undergo remission in the presence of high baseline BMI and elevated C-peptide levels. A nationwide register-based cohort study from Sweden revealed that the chance of achieving complete remission correlated negatively with the duration of DM, insulin treatment, age, and HbA1c at baseline. Remission rates were higher among males and those having higher BMI at baseline[54]. Other reported predictors of remission in different studies are higher liver enzymes, higher white blood cell count, serum creatinine, serum LDL cholesterol and absence of long acting insulin[59,62-64].

Visceral adipose tissue is closely linked to insulin resistance and has been explored as a marker of remission[65-67]. BMI and waist circumference however might underestimate the amount of visceral adiposity in Asian population[68,69]. VFA as assessed by

magnetic resonance imaging was associated with a higher chance of remission in candidates with BMI < 35 kg/m^2[57]. Visceral adiposity index (VAI) calculated from waist circumference, BMI, serum triglycerides and high-density lipoprotein is a validated marker of visceral fat content[70]. In a study from China, VAI was able to reliably predict remission in persons with BMI < 35 kg/m^2[71]. The estimates of visceral adiposity might be potentially better pointers of insulin resistance than anthropometric parameters in Asians with lower BMI and hence may more reliably predict probability of remission. Further validation of this hypothesis is however needed in larger and long-term studies.

Scoring systems for remission: Several scoring systems have been proposed as predictors of DM remission following metabolic surgery. The ABCD scoring system devised by Lee *et al*[72], incorporated age at surgery (A), baseline BMI (B), C-peptide level (C), and duration of DM (D). The DiaRem score was suggested by Still *et al*[73], and includes age, insulin use, HbA1C level, and type of anti-diabetic medication. The IMS score categorizes patients into three stages of severity based on the preoperative number of DM medications, insulin use, duration of DM, and glycemic control (HbA1c < 7%). The system also provides recommendations on the type of procedure (RYGB or LSG) for each severity stage based on each procedure's efficacy and risk-benefit ratio [74]. Though one analysis suggested that the ABCD score had better predictive efficacy as compared to the IMS score and DiaRem score, the committee does not acknowledge one scoring system's superiority above the other in the absence of evidence from large multicenter studies[75,76]. ACF scoring system is another recently reported model that utilizes the three variables: age, C-peptide area under curve, and FPG to predict remission[77].

PREOPERATIVE ASSESSMENT AND OPTIMIZATION OF GLYCEMIC STATUS

Recommendation 10

The initial preoperative assessment should include a comprehensive medical, psychosocial and drug history, along with physical examination. Appropriate laboratory tests should be done to assess glycemic control. These tests should include FPG, postprandial glucose, and HbA1c in all cases and self-monitoring of blood glucose and/or continuous glucose monitoring system in selected cases. Estimation of serum C-peptide should be done to assess the scope for the remission of DM (E).

Recommendation 11

A glycemic target of HbA1c < 7% before surgery is a reasonable goal. Medical nutrition therapy, physical exercise, and pharmacotherapy should be optimally integrated to attain that goal (E). Pharmacological agents known to induce weight loss, such as sodium-glucose co-transporter-2 inhibitors and glucagon-like peptide-1 receptor agonists, should be considered as part of the treatment armamentarium whenever feasible. Drugs known to cause weight gain, such as sulfonylureas and thiazolidinediones, should be avoided as long-term therapeutic strategy if possible. The perioperative risks of deranged glycemic control *vs* benefits of early metabolic surgery have to be assessed on a case-to-case basis if glycemic control cannot be attained preoperatively despite optimal medical treatment. If a strategy of restricting calories with meal replacement therapy is employed in the preoperative weeks, the anti-diabetic medications would need to be reduced to prevent hypoglycemia (E).

Recommendation 12

After admission, most non-insulin based therapies should be stopped, and the patient should be transitioned to insulin as per institutional practice. Severe degrees of hyperglycemia will require intravenous insulin infusion. Target glucose of 100 to 180 mg/dL (5.5-10 mmol/L) is acceptable in the perioperative period (E).

Discussion

Glycemic target: Table 5 summarizes the recommended evaluation in individuals with DM before metabolic surgery. Inadequate glycemic control in the preoperative period is associated with increased 1-year mortality, wound complications, infective complications, and extended hospital stay[78-81]. Pre-surgery deranged glycemic status and medication usage, including insulin and the number of drugs required to achieve

Table 5 Preoperative evaluation before metabolic surgery in individuals with diabetes and obesity

System	Essential evaluation	Conditional evaluation	Comments
History and physical examination	Detailed evaluation along with drug history	-	-
Glycemic	FPG, PPG, HbA1c, Fasting serum C-peptide	SMBG; CGMS	HbA1c < 7% is a reasonable target, higher targets may be acceptable in long-standing diabetes; SMBG and/or CGMS in patients on insulin
Cardiovascular	BP: Fasting lipid Profile; ECG: Cardiovascular risk assessment with a validated risk prediction model[1]	Transthoracic echocardiography (in cases with unexplained dyspnea and known cases of heart failure, especially with recent changes in clinical status); If risk ≥ 1%,[2] functional status assessment. Poor (< 4 METs) or unknown functional capacity - exercise or pharmacological stress echocardiography or radionuclide MPI	Target BP < 140/90; Abnormal results in a stress test should be managed according to current clinical practice guidelines. Patients with underlying cardiac abnormalities should undergo a formal cardiology consultation before surgery
Pulmonary	Smoking history. Screening for OSA by a clinical scoring tool[3]. Risk assessment for VTE during perioperative period by a validated method[4]	Pulmonary function test in presence of intrinsic pulmonary disease; Overnight polysomnography if indicated from results of scoring tool. ABG for $PaCO_2$ estimation and venous bicarbonate in cases of OSA to rule out OHS	Structured tobacco cessation program if applicable
Gastrointestinal	-	UGIE to be considered routinely before LSG. Conditional for other procedures; H pylori detection and eradication	
Hepatic	LFT	Abdominal USG if LFT deranged or symptomatic biliary disorder. Use of Noninvasive scoring systems[5] can be considered. Liver elastography; Three-dimensional magnetic resonance elastography; Intraoperative liver biopsy	The strategy to diagnose NAFLD in bariatric patients is not defined. Variations of liver elastography such as transient elastography, 2-D shear wave elastography, and ARFI can be better modalities in severely obese patients. Intraoperative liver biopsy is the gold standard, but its specific indications are not clear
Renal, electrolytes, uric acid	Serum creatinine; eGFR[6]; Urinary albumin-creatinine ratio	Electrolytes in presence of CKD or drugs known to cause electrolyte imbalance. Uric acid if there is past history of gout	Serum potassium should be measured if on ACE inhibitors, ARBs, or diuretics
Nutritional	Nutritional assessment by a dietitian. Complete blood count, serum ferritin, serum iron, TIBC, and TS. Serum vitamin B12, folate. Serum calcium, 25(OH)D	Serum C-reactive protein if anemia of chronic inflammation is suspected. Serum methylmalonic acid and homocysteine in cases of low normal vitamin B12 and folate levels with high index of suspicion. Serum copper, zinc, and selenium; fat soluble vitamins such as vitamin A, E and K can be considered before malabsorptive procedures	Serum or urinary N-telopeptide, bone-specific alkaline phosphatase, and bone mineral density can be considered if osteoporosis is suspected especially in postmenopausal women
Endocrine	-	Thyroid profile if there is a past history of thyroid dysfunction, goiter or symptoms suggestive of thyroid disorder. ONDST, 24-h urinary free cortisol, or 11-pm salivary cortisol if there is suspicion of endogenous Cushing's syndrome	Evaluation of syndromic or monogenic obesity on case-by-case basis
Reproductive	-	Total and bioavailable testosterone and USG of the pelvis if PCOS is suspected. LH, FSH, and testosterone (total) if hypogonadism is suspected in males	Women should avoid pregnancy if planned for surgery. Pregnancy should be avoided for 12-18 mo after surgery
Psychological	Behavioral and psychosocial evaluation	-	-

[1]*e.g.*, Revised Cardiac Risk Index, Obesity surgery mortality risk score, Longitudinal Assessment of Bariatric Surgery consortium risk stratification system, metabolic acuity score, *etc.*

[2]Estimated perioperative mortality risk or major adverse cardiovascular risk of ≥ 1%.

[3]STOP-BANG questionnaire or Berlin questionnaire.

[4]*e.g.*, venous thromboembolism risk assessment tool by Fink *et al*[130].

[5]Non-alcoholic steatohepatitis clinical scoring system, AST to platelet ratio index, FIB-4 index, non-alcoholic fatty liver disease fibrosis score, BARD score and Forns index.

[6]By Chronic Kidney Disease Epidemiology Collaboration formula.

25(OH)D: 25-hydroxyvitamin D; ABG: Arterial blood gas; ACE: Angiotensin converting enzyme; ARB: Angiotensin II receptor blocker; ARFI: Acoustic radiation force impulse shear wave imaging; CGMS: Continuous glucose monitoring system; eGFR: Estimated glomerular filtration rate; FPG: Fasting plasma glucose; FSH: Follicle-stimulating hormone; *H. pylori*: *Helicobacter pylori*; HbA1c: Glycated hemoglobin; LFT: Liver function test; LH: Luteinizing hormone; MET: Metabolic equivalent; MPI: Myocardial perfusion imaging; ONDST: Overnight dexamethasone suppression test; OSA: Obstructive sleep

apnea; PCOS: Polycystic ovary syndrome; PPG: Post-prandial glucose; SMBG: Self-monitoring of blood glucose; TIBC: Total iron-binding capacity; TS: Transferrin saturation; UGIE: Upper gastrointestinal endoscopy; USG: Ultrasonography; VTE: Venous thromboembolism.

euglycemia, negatively correlate with the chance of long-term remission of DM following metabolic surgery[61,73,74,76]. Only a few studies, however, have specifically assessed the role of preoperative glycemic control to short-term postoperative outcomes. The clinical practice guidelines by the AACE/TOS/ASMBS/OMA/ASA suggest an HbA1C target of 6.5% to 7.0% or less before surgery, and peri-procedure blood glucose levels of 80 to 180 mg/dL (4.4–10 mmol/L). In the presence of advanced microvascular or macrovascular complications, or comorbidities, or long duration of DM, they recommended an HbA1C target between 7% and 8% [33]. An interprofessional bariatric glycemic optimization clinic-based study analyzing 70 patients, was able to lower HbA1C from a mean level of 9.0% ± 1.2% to ≤ 7.5% in 75% of patients before surgery in 5 mo[82]. In a retrospective review of 468 patients who had undergone RYGB, higher pre-surgery HbA1c (> 6.5%) was associated with an increased chance of postoperative hyperglycemia. These patients also had a greater risk of wound infection and acute renal failure[83].

A RCT of 34 patients with a mean A1C of 10% at baseline, did not show any differences in the length of stay or surgical complications in the two arms of optimized (HbA1c-8.4%) *vs* non-optimized (HbA1c–9.7%) glycemic therapy. This was the only RCT that attempted to identify the impact of two different glycemic strategies, but had shortcomings such as a narrow margin between achieved HbA1c (> 8% in both arms) and small sample size. Another drawback was both the arms were offered the same dietary and glycemic interventions for the preceding 2 wk immediately before surgery [84]. A target glucose of 100 to 180 mg/dL (5.5-10 mmol/L) should be the perioperative period goal[85]. The approach to achieve this target should be guided by institutional policy. Intravenous insulin as per protocol should be administered in cases of severe hyperglycemia[86].

Pre-operative calorie restriction for two to four weeks has been conventionally practiced in many bariatric centers. Though the methods have been very variable, these practices might warrant adjustment of anti-diabetic medications[87,88].

PREOPERATIVE ASSESSMENT AND OPTIMIZATION OF CARDIOVASCULAR STATUS

Recommendation 13

The target blood pressure (measured by appropriately sized cuff) before surgery is < 140/90 mmHg. Angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), thiazide diuretics, or dihydropyridine calcium channel blockers (CCBs) are the preferred agents, and multiple drug classes are usually necessary to accomplish blood pressure goals (a combination of ACE inhibitors and ARBs to be avoided) (A). Patients already on beta-blocker should be continued on the same. Initiating a beta-blocker in the preoperative phase is controversial and should be individualized after estimating risk *vs* benefit (B).

Recommendation 14

A fasting lipid profile should be done in all patients. Lipid-lowering therapy as per current recommendations should be initiated (A).

Recommendation 15

A resting 12-lead electrocardiogram (ECG) should be obtained before metabolic surgery. The ECG, other than serving as a baseline for comparison in any subsequent cardiac adverse events, can provide clues regarding left ventricular hypertrophy, possible ischemia (Q waves, ST-segment depression), and bundle branch blocks, arrhythmia, and QTc prolongation (B).

Recommendation 16

Assessment of left ventricular function by resting transthoracic echocardiography should be undertaken in patients with unexplained dyspnea, and known cases of heart failure, especially with recent changes in clinical status. Right ventricular hypertrophy in echocardiography can be indicative of pulmonary hypertension. Valvular

abnormalities and other structural lesions can also be detected by echocardiography (B).

Recommendation 17

A cardiovascular risk assessment is recommended before surgery. Several risk prediction models to assess perioperative cardiac risk have been suggested and validated in obese individuals. Individuals at an elevated risk (1% or more) of a major adverse cardiac event (MACE) during the perioperative period and having a poor (< 4 metabolic equivalents [METs]) or unknown functional capacity should undergo further risk stratification with exercise or pharmacological stress echocardiography, or radionuclide myocardial perfusion imaging to assess for myocardial ischemia. Individuals with a normal stress test can proceed to surgery, whereas those with an abnormal result should be managed according to the current clinical practice guidelines. Those with underlying cardiac abnormalities should undergo a formal cardiology consultation before surgery (E).

Discussion

Blood pressure and lipids: The target blood pressure recommended by ADA in individuals with DM is less than 140/90 mmHg. Lower targets such as 130/80 can be pursued for individuals at high risk of cardiovascular disease if that goal can be attained by reasonable therapeutic means[89]. Meta-analyses have demonstrated equivalent efficacy of ACE inhibitors or ARBs (to be avoided together), thiazide diuretics, or dihydropyridine CCBs in reducing cardiovascular outcomes, and any of these can be used as a first-line agent[90,91]. Most individuals, however, will require multiple agents for normalization of blood pressure[89]. As per the American College of Cardiology (ACC)/American Heart Association (AHA) recommendations published in 2014, beta-blockers should be continued in those who have been receiving them for long duration. The risks and benefits of initiating beta-blockers before surgery should be individualized according to the clinical situation[92]. Lipid-lowering therapy should be initiated as per the current practice guidelines[93].

12-lead ECG and echocardiography: A 12-lead ECG should be obtained before metabolic surgery. It acts as a reference against which the postoperative changes can be compared. Also, arrhythmias, pathological Q-waves, LV hypertrophy, ST depression, QTc interval prolongation, and bundle-branch blocks can provide useful clues, but the prognostic utility of an ECG to predict the perioperative cardiovascular outcome is limited[92,94]. The ACC/AHA guidelines recommend assessment of LV function by echocardiography in patients with dyspnea of unknown origin and for patients with heart failure with worsening dyspnea or recent change in clinical status [92].

Cardiac risk prediction models: Several risk prediction models have been proposed to estimate the perioperative risk of MACE in individuals undergoing non-cardiac surgery[95-98]. The most commonly used scoring system is the Revised Cardiac Risk Index, which incorporates the following variables as predictors of perioperative cardiac risk: high-risk surgery, history of ischemic heart disease, history of CHF, creatinine > 2 mg/dL, cerebrovascular disease, and DM requiring insulin. The 30 d risk of death, myocardial ischemia, or cardiac arrest is 0.4% if none of the factors are present. The presence of one predictor pertains to a risk of 0.9% for these events, two predictors carry a 6.6% risk, and three or more factors correlate with an 11% risk[99]. Bariatric surgery itself is considered as an intermediate to high-risk non-cardiac surgery, and the presence of any other additional factor will warrant further assessment[100].

Scoring systems such as obesity surgery mortality risk score (OS-MRS) for bariatric surgery have also been validated. The OS-MRS assigns one point each to the following five risk factors: age ≥ 45 years, male sex, BMI ≥ 50 kg/m^2, hypertension, and known risk factors for pulmonary embolism (previous thrombosis, pulmonary embolism, inferior vena cava filter *in situ*, a history of right heart failure or pulmonary hypertension and obesity-hypoventilation syndrome)[101]. The mortality rate in class A (score 0 to 1) was 0.26%, in class B (score 2 or 3) was 1.33%, and in class C (score 4 or 5) was 4.34%[102]. Other methods that have been used to stratify surgical risk in obese patients include the Longitudinal Assessment of Bariatric Surgery consortium risk stratification system, the metabolic acuity score, and a nomogram for assessing surgical complications in bariatric surgery[103-105]. Our committee recommends individualized evaluation of candidates as per recommended practices before undergoing surgery, if they have an estimated (by general or obesity specific scoring

system) perioperative mortality or MACE risk ≥ 1%. Further studies are required to validate the optimal prediction model to stratify perioperative risk in individuals undergoing metabolic surgery for obesity and DM.

Approach for cardiovascular evaluation and management: Our committee endorses the approach suggested in the 2014 ACC/AHA guideline of perioperative cardiovascular evaluation and management of patients undergoing non-cardiac surgery. In all cases at elevated risk (estimated perioperative mortality risk or MACE risk ≥ 1%), assessment of the functional status of the patient should be undertaken. In case the patient can perform ≥ 4 METs of activity (can walk up a flight of steps or a hill or walk on level ground at 3 to 4 mph), additional tests are usually not recommended. For those whose functional capacity is lower or unknown, additional stress testing may be indicated if it will influence perioperative care[92]. Assessment of functional capacity might not be possible in many patients with obesity for unrelated reasons such as osteoarthritis. Pharmacological stress testing may be warranted in such cases. Both obesity and DM are substantial risk factors for cardiovascular disease. In many situations, the strategy to assess cardiac risk has to be individualized in consultation with the cardiologist, specifically when proper assessment of the patient's functional status cannot be performed. Those with pre-existing cardiological disease must undergo a formal cardiology consultation before metabolic surgery[33].

PREOPERATIVE EVALUATION AND OPTIMIZATION OF PULMONARY FUNCTION

Recommendation 18

A structured tobacco cessation program should be employed for patients who smoke cigarettes before undergoing surgery (E).

Recommendation 19

Pulmonary function test (PFT) or spirometry is not routinely indicated. Definitive evidence that PFT can predict postoperative pulmonary complications is lacking, and the testing should be restricted for those with an intrinsic pulmonary disease where the findings would alter the management (E).

Recommendation 20

Untreated OSA increases the risk of perioperative complications. Considering the high prevalence of undiagnosed OSA in severely obese individuals, screening for OSA by a clinical scoring tool such as the STOP-BANG questionnaire (or Berlin questionnaire) should be performed. The gold standard test to confirm the diagnosis of OSA in suspected cases is overnight polysomnography (PSG). The use of continuous positive airway pressure (CPAP) in the preoperative period for treatment of moderate to severe OSA is recommended to reduce the risk of perioperative pulmonary complications (B).

Recommendation 21

OHS often coexist with OSA in severely obese individuals, and the presence of OHS should be ruled out in all patients diagnosed to have OSA. Arterial blood gas analysis for $PaCO_2$ estimation along with measurement of venous HCO_3^- (cut off 27 mmol/L) can be used to establish the diagnosis of OHS. Institution of positive airway pressure therapy and lifestyle modification is recommended for patients diagnosed to have OHS (B).

Recommendation 22

A risk assessment for possibility of development of venous thromboembolism (VTE) in the perioperative period should be undertaken. The possible risk factors for VTE include prior VTE, higher BMI, age, gender, immobility, use of hormone therapy, OHS, pulmonary hypertension, venous stasis disease, operative time, and procedure type and approach (B). There are insufficient data to recommend a uniform strategy to prevent VTE complications. The standard recommendations are mechanical compression devices with early ambulation in addition to chemoprophylaxis (B). There is inadequate evidence to recommend prophylactic placement of inferior vena cava (IVC) filters to prevent pulmonary embolism and it should be applied under very selected circumstances (E).

Discussion

Smoking and tobacco cessation: Smoking increases the risk of postoperative morbidity following metabolic surgery. Smoking is associated with an increased risk of organ space infection, prolonged intubation, reintubation, pneumonia, sepsis, shock, and longer length of hospital stay[106]. In a recent systemic review, smoking during a year before undergoing surgery, was an independent risk factor for higher 30 d mortality and major postoperative complications, particularly wound and pulmonary complications[107]. Perioperative tobacco cessation reduces the chance of surgery-related morbidities[108]. A structured program is more effective than general advice[109].

PFT: The utility of PFT to predict postoperative complications is uncertain. In a prospective study of 485 patients who underwent laparoscopic metabolic surgery, abnormal spirometry in the preoperative period was associated with a three-fold risk of postoperative complications[110]. In a retrospective analysis of 602 patients, abnormal spirometry before surgery was shown to correlate with the risk of postoperative pulmonary complications only in those with OSA[111]. In another retrospective cohort of 146 severely obese BPD candidates, the logistic regression model suggested that the preoperative PFT could not predict respiratory complications after surgery[112].

OSA: OSA is a common comorbidity of severe obesity, with the reported prevalence of close to 80% in patients planned for metabolic surgery[113-116]. Untreated OSA increases the risk of postoperative complications[117,118]. We endorse the consensus guidelines by de Raaff *et al*[119]. regarding screening and management of OSA before metabolic surgery. A clinical scoring tool to screen for OSA should be used in all patients planned for surgery. The STOP-Bang Questionnaire's sensitivity to detect mild and severe OSA was highest, while the STOP Questionnaire had the highest sensitivity to predict moderate OSA[120]. Berlin questionnaire can also be used to screen, but Epworth Sleepiness Scale is not recommended[119]. Overnight PSG is the gold standard to confirm the diagnosis of OSA. Simpler portable devices that can analyze a limited range of variables, known as type 3 portable sleep monitoring (as per definition of the American Academy of Sleep Medicine) can be used if there is a high pretest probability for moderate to severe OSA[119,121]. CPAP usage in the perioperative phase decreases the chance of pulmonary complications and is recommended for treatment of moderate to severe OSA[119,122].

OHS: OHS is defined as the triad of obesity (BMI > 30 kg/m^2), daytime hypoventilation, and sleep-disordered breathing in the absence of an alternative neuromuscular, mechanical, or metabolic explanation for hypoventilation. The prevalence of OHS is 20%-30% among individuals with obesity and OSA[123]. In a recently published study, the prevalence of OHS in a bariatric cohort of 1718 patients was 68%[124]. OHS should be ruled out in patients diagnosed with OSA by measuring serum HCO_3^- or arterial blood gas analysis. Elevated serum HCO_3^- (> 27 mmol/L) and/or increased $PaCO_2$ (> 45 mmHg) are indicative of OHS[125]. Institution of positive airway pressure therapy along with lifestyle modification is recommended for patients diagnosed to have OHS[119,126].

VTE: Individuals with obesity are at an increased risk of VTE, though the overall rate following metabolic surgery has been reported to be < 1%[127-129]. A preoperative risk assessment model to stratify candidate by VTE risk was devised by Fink *et al*[130] using the following variables: procedure type, patient history of VTE, male sex, BMI, age, and operative time > 3 h. By this scheme, 97% were classified into low-risk groups with a predicted VTE risk of < 1%. The medium-risk group had an estimated VTE risk of 1%-4%, and the high-risk group had > 4% - 30 d VTE event rate[130]. Other tools to assess DVT risk have also been proposed[131]. Further risk factors for VTE include immobility, known hypercoagulable condition, OHS, pulmonary hypertension, venous stasis disease, hormonal therapy, and transfusion[129,130,132,133]. We endorse the ASMBS recommendations for VTE prophylaxis in those undergoing metabolic surgery. All candidates are at moderate to high risk of VTE and require mechanical prophylaxis and early ambulation. Additional chemoprophylaxis with low molecular weight heparin should be considered unless contraindications arise from bleeding tendency or other causes[134]. Placement of IVC filter is not routinely indicated[134,135].

GASTROINTESTINAL EVALUATION

Recommendation 23

Preoperative upper gastrointestinal (GI) endoscopy can be considered before performing metabolic surgery, though conclusive evidence supporting its routine usage is lacking (C). Because of worsening of previous GERD and risk of development of new-onset GERD on long-term follow-up after LSG, it is recommended that upper GI endoscopy should be performed in candidates for LSG (B).

Recommendation 24

There is inadequate evidence to support or refute in favor of routinely performing tests to detect and treat *Helicobacter pylori* during preoperative evaluation.

Discussion

Upper gastrointestinal endoscopy: There is a lack of consensus regarding the utility of routine upper GI endoscopy before surgery[33,136]. Two meta-analyses explored the benefits of preoperative endoscopy[137,138]. In the meta-analysis by Parikh *et al*[137] (n = 6112), endoscopic findings were normal in the majority (92.4%), and only 7.6% had abnormalities that delayed or altered surgery[137]. In the meta-analysis by Bennett *et al*[138] (n = 12261), endoscopic findings necessitated a change in surgical management in 7.8% and medical management in 2.5% (after excluding *H. pylori*). The authors concluded that preoperative upper GI endoscopy in average-risk, asymptomatic individuals should be considered optional as the proportion of endoscopies with findings that resulted in alteration in management was low[138]. In two recent studies, treatment strategy was changed only in a small percentage of patients based on endoscopic findings[139,140].

GERD: GERD is increasingly recognized as a long-term complication of LSG and has been considered a relative contraindication[141]. A meta-analysis evaluating the outcome of 10718 patients after LSG found that 19% had increased symptoms and 23% developed new-onset GERD after surgery. The long-term prevalence of esophagitis was 28%, and that of Barrett's esophagus (BE) was 8%[142]. A significant percentage of patients detected to have GERD on endoscopy are asymptomatic and thus were diagnosed only on routine screening[143]. The prevalence of GERD in bariatric candidates was not different between those with or without DM[144]. Our panel recommends upper GI endoscopy in patients planned for LSG to rule out symptomatic and asymptomatic GERD. Dedicated studies to assess the evolution of GERD after LSG are required.

Gastric lesions: A recent meta-analysis scrutinized gastric lesions that requires subsequent endoscopic monitoring after surgery. Atrophic gastritis was detected in 2.64%, and intestinal metaplasia in 2.7%[145]. Lesions such as atrophic gastritis, intestinal metaplasia, or gastrointestinal stromal tumor mandate endoscopic monitoring post-surgery. LSG should be considered in preference to RYGB in the presence of these conditions. However, the prevalence of these lesions is negligible, and it is not clear whether routine preoperative endoscopy will have a significant role.

***H. pylori*:** There is a lack of consensus regarding the utility of detecting and eradicating *H. pylori* before metabolic surgery[136]. A meta-analysis of seven studies with 255435 patients revealed that rates of bleeding, leak, length of hospital stay, and weight loss were similar between *H. pylori* positive and negative groups. Marginal ulceration following RYGB was the only outcome that correlated with its presence [146]. Another meta-analysis demonstrated that eradication of *H. pylori* decreased the risk of marginal ulceration, but the rates still remained high (1.5%-18.8% following eradication *vs* 0.5%-31.2% in the non-eradicated group). The authors acknowledged the methodological limitation in many of the studies included in the meta-analysis [147]. Efficacy of *H. pylori* treatment in preventing complications (especially after RYGB) needs further assessment in well-designed trials.

HEPATIC EVALUATION

Recommendation 25

All candidates for surgery should be investigated for NAFLD. There is no consensus about the methodology for diagnosis of NAFLD. Liver function test (LFT) should be

performed routinely before surgery. Abdominal ultrasonography (USG) is recommended if LFT is deranged or symptomatic biliary disorder is suspected. Evidence to support routine imaging of the liver during preoperative evaluation is lacking (E).

Recommendation 26

Several noninvasive scoring systems have been proposed to assess the risk of fibrosis in the bariatric population. However, more evidence is required before a particular strategy can be recommended for clinical application. Liver elastography can be considered in those with suspected NAFLD, but diagnostic accuracy is limited in severe obesity. The gold standard for diagnosing NAFLD is intraoperative liver biopsy, but the clinical strategy to identify patients who will benefit from the biopsy has to be formulated through well-structured studies (E).

Discussion

NAFLD is present in up to 81% of patients undergoing metabolic surgery. The global prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) in T2DM were reported to be 55.5% (95%CI: 47.3-63.7) and 37.3% (95%CI: 24.7-50.0) respectively in a meta-analysis[150]. The therapeutic options for NAFLD are limited and metabolic surgery is the only modality that has consistently demonstrated benefit[149,151-153]. However the modalities and clinical strategy to diagnose and follow these patients are not clearly defined[154-157]. Even though the sensitivity of USG to detect NAFLD is high, but its low specificity in obese individuals remains a drawback[148,158]. The noninvasive fibrosis scores that have been commonly studied in the bariatric population include NASH clinical scoring system NCS[159], aspartate aminotransferase to platelet ratio index[160], fibrosis-4 index[161], NAFLD fibrosis score[162], BARD score[163], and Forns index[164]. The scoring systems were able to predict fibrosis in some but not in all studies[155,165,166]. Their usefulness in detecting fibrosis before surgery requires validation in more extensive studies. Transient elastography, two-dimensional shear wave elastography, and acoustic radiation force impulse shear wave imaging reliably predicted advanced fibrosis in bariatric candidates in small studies[167-169]. Three-dimensional magnetic resonance elastography is a promising modality to detect NASH and has demonstrated a sensitivity of 67% and specificity of 80%[170]. Intraoperative liver biopsy remains the gold standard for the diagnosis of NASH in bariatric candidates. However, it is associated with a small increase in the rate of complications[149]. The morphology of the liver can be visualized during surgery and can provide a clue regarding necessity for biopsy[158].

Obesity and DM are associated with high prevalence of NAFLD, often with significant fibrosis[151,158]. Metabolic surgery does improve outcomes related to NAFLD in a significant proportion of patients[152-154]. Post-surgery hepatology follow-up to assess the risk of progression to cirrhosis is recommended in these groups. None of the imaging modalities and noninvasive scoring systems have convincing evidence to support their routine clinical application. Intraoperative liver biopsy provides a reliable way to diagnose and assess the severity of NAFLD, but it is currently unclear what criteria should be applied to identify patients who will benefit from biopsy.

ASSESSMENT OF RENAL FUNCTION, ELECTROLYTES, AND URIC ACID

Recommendation 27

Individuals with DM planned for metabolic surgery should undergo estimation of spot urinary albumin-creatinine ratio (ACR). Serum creatinine measurement along with the assessment of estimated glomerular filtration rate (eGFR) is also recommended (E).

Recommendation 28

In patients on diuretics, ACE inhibitors, or ARBs, serum potassium levels should be obtained. Studies to support the measurement of electrolytes on a routine basis before surgery are lacking. Clinical factors, especially the presence of chronic kidney disease (CKD) and drug history, usually indicate whether assessment of other electrolytes is necessary (E).

Recommendation 29

Serum uric acid should be measured in individuals with a history of gout, and prophylactic treatment of acute gouty arthritis should be considered in these patients (E).

Discussion

Calculation of eGFR in severe obesity: Both DM and obesity are leading causes of the development and progression of CKD[171-173]. The ADA recommends estimating urinary ACR and serum creatinine (along with eGFR) in individuals with DM annually. Serum potassium should be measured in individuals receiving ACE inhibitors, ARBs, or diuretics[174]. Calculation of eGFR is usually done using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula[175]. The CKD-EPI formula using creatinine tends to overestimate eGFR in bariatric patients both before and after surgery, while the cystatin C CKD-EPI equation tends to underestimate it[176]. The errors in using the standard equations in severely obese subjects arise from changes in the body surface area (error of indexing), alteration in serum creatinine and cystatin C levels, and obesity-induced glomerular hyperfiltration [177]. The combined equation CKD-EPIcreat-cyst using serum creatinine and cystatin C values reliably predicted eGFR in severe obesity both before and after surgery but further validation in larger cohorts is needed[176,178,179].

Reno-protective effects of metabolic surgery: Metabolic surgery is a promising reno-protective strategy in obesity, even without DM[180,181]. In a study of 737 subjects, remission of DM 5 years after surgery was associated with a lower risk of moderate or severe albuminuria but did not result in stabilization of eGFR[182]. A meta-analysis of 23 cohort studies with 3015 subjects reported a significant fall in serum creatinine level and proteinuria after surgery. The subgroup of patients with hyperfiltration and CKD also showed improvement in eGFR after 6 mo[183].

Precautions in CKD: The weight-lowering response following surgery and chances of DM remission may be diminished in CKD stages 4 and 5[182,184,185]. Decreased eGFR before metabolic surgery also correlated with a higher chance of surgical site complications, infections, cardiovascular events, and clotting disorders in the postoperative period. However, the overall number of adverse events across all stages were low[186,187].

Renal stone disease: Another renal adverse event with the potential for causing acute and chronic kidney damage is hyperoxaluria occurring after malabsorptive procedures [188-190]. A meta-analysis of 11 observational studies demonstrated that RYGB increases the risk of hyperoxaluria and renal stone formation[190]. Age, history of urinary tract infection, and renal stone disease correlate with a higher chance of new stone formation after surgery[191,192]. History of nephrolithiasis mandates close urological follow-up post-surgery.

Hyperuricemia: Obesity and hyperuricemia are closely interrelated and often coexist [193,194]. A meta-analysis of 20 studies with 5233 participants reported that the mean serum uric acid before surgery was 6.5 mg/dL. Metabolic surgery was followed by a transient elevation in serum uric acid in the first month, followed by a fall from the third month. The long-term incidence of gout was decreased[195]. There is a higher possibility of acute gout in the postoperative phase, and high-risk patients should be considered for prophylactic uric acid lowering medications before surgery[196,197].

NUTRITIONAL ASSESSMENT AND OPTIMIZATION

Recommendation 30

A preoperative nutritional assessment by a dietitian with expertise in bariatric counselling should be considered. Current macronutrient and micronutrient intake pattern should be evaluated. Medical nutrition therapy to optimize glycemic control before surgery should be reinforced. The candidate should be educated about dietary and lifestyle changes required after the surgery (E).

Recommendation 31

The low-grade chronic inflammation associated with obesity, destabilizes the iron homeostasis and predisposes to iron deficiency, and decreases iron bioavailability. A

complete blood count, serum ferritin, serum iron, total iron-binding capacity, and transferrin saturation (TS) is recommended during preoperative work-up. In presence of chronic inflammation denoted by serum CRP > 8 mg/L, serum ferritin loses specificity as an indicator of iron deficiency, and other markers like serum iron and TS should be used to define iron deficiency. Iron deficiency should be treated by oral iron supplementation. Parenteral iron should be considered if oral treatment is not tolerated or if early correction is needed (B).

Recommendation 32

Estimation of vitamin B12 and folate levels are recommended during pre-operative work up. In low-normal cases with high index of suspicion, measurement of serum methylmalonic acid and homocysteine levels can be considered. Vitamin B12 deficiency should be corrected by parenteral administration in symptomatic cases, whereas oral therapy is sufficient in asymptomatic individuals. Folic acid deficiency should be treated by oral supplements (B).

Recommendation 33

Vitamin D deficiency (VDD) is prevalent in individuals with obesity. Estimation of serum calcium and 25-hydroxyvitamin D (25(OH)D) is recommended before surgery. Serum parathyroid hormone (PTH) assessment can be considered in patients with VDD. Vitamin D should be replaced orally if VDD is present (B).

Recommendation 34

Estimation of copper, zinc, and selenium; and fat-soluble vitamins such as vitamin A, E and K can be considered before malabsorptive procedures.

Discussion

Nutritional counselling: A meta-analysis of three RCTs revealed that there was inadequate data to support or refute preoperative nutritional counselling[198]. A review on the same topic suggested that preoperative medical weight management strategies failed to achieve consistent benefits probably because of lack of dedicated trials[199]. Even then, nutritional counselling is safe, requires minimal resources, helps to create a rapport with the bariatric team and prepares the patient for necessary lifestyle modifications required around and after the surgery. It is an important adjunct to comprehensive bariatric care and is recommended by most guidelines[33,136,200].

Anemia and iron deficiency: Micronutrient deficiency is common among candidates of metabolic surgery and worsens further during follow-up[201,202]. The prevalence of anemia and iron deficiency ranges from 6.1%-22% and 5.7%-24% respectively, in metabolic surgery candidates[203-207]. Low-grade inflammation associated with obesity can interfere with the intestinal absorption and iron utilization in the bone marrow[208,209]. Iron deficiency is characterized by serum ferritin < 30 ng/mL. CRP concentration above 8 mg/L is suggestive of overt inflammation, while levels between 3 to 8 mg/L conventionally signify subclinical inflammation[210]. In the presence of CRP > 8 mg/L, serum ferritin up to a concentration of 100 ng/mL indicates iron deficiency[211]. Serum CRP > 8 mg/L, serum ferritin > 100 ng/mL, and TS < 20% denotes anemia of chronic disease[212].

CRP > 5 mg/L, often associated with mild inflammation present in obesity, can alter iron metabolism[212,213]. Laboratory assessment of iron deficiency should be done at least 1 mo before surgery so that adequate time for replenishment of stores is available [214]. Iron supplementation is usually done through oral formulations, but parenteral preparations may be necessary if oral iron is not tolerated or if quick response is required[213,214]. Parenteral iron therapy has the advantages of rapid replenishment of iron stores before surgery and overcomes the uncertainty of absorption, compliance, and tolerability associated with oral therapy[214]. Systemic studies that investigate the benefits of parenteral iron therapy before metabolic surgery are required.

Vitamin B12 and folic acid deficiency: Vitamin B12 deficiency has been reported in up to 23% of candidates of metabolic surgery[206,215-218]. Additionally, metformin is known to cause deficiency of vitamin B12 in T2DM[219,220]. Folic acid deficiency is common among patients planned for metabolic surgery and is reported in up to 28% of cases[217,221,222]. Estimation of serum vitamin B12 and folate levels are recommended before surgery[33,223]. Low vitamin B12 should be treated by parenteral administration in megaloblastic anemia, neuropathy, or in presence of other deficiency symptoms[33,200,223-225]. A systemic review by Smelt *et al*[226] suggested

that daily oral administration of 350 μg of vitamin B12 corrects low levels in most cases. The guideline by AACE/TOS/ASMBS/OMA/ASA suggests oral vitamin B12 at a dose between 350 to 1000 μg every day. Alternatively it can be administered by nasal route[33]. Oral folic acid supplementation should be initiated to correct deficiency[33,223]. Measurement of serum methylmalonic acid and homocysteine to assess for functional deficiency can be considered in cases with low-normal levels but high-index of suspicion, though such a strategy has not been validated[33,224,227,228].

VDD: Systematic reviews and meta-analyses have suggested that VDD is common in obese individuals and candidates of metabolic surgery[229-232]. Most guidelines recommend routine measurement of serum calcium and 25(OH)D before surgery[33,201,223,224]. Estimation of serum PTH, serum or urinary N-telopeptide, bone-specific alkaline phosphatase, and bone mineral density can be considered if osteoporosis is suspected (especially in postmenopausal women)[224,233]. VDD should be corrected before surgery but there is no consensus on the exact dosage with guidelines recommending between 3000 IU daily to 50000 IU one to three times weekly[234].

Other trace elements: Malabsorptive procedures can cause deficiency of trace elements like zinc, copper and selenium and fat soluble vitamins (vitamins A, D, and E)[201,235-237]. Some of the guidelines suggest their preoperative measurement[33,223,224].

ENDOCRINE AND REPRODUCTIVE FUNCTION ASSESSMENT

Recommendation 35

Case-by-case decision depending on the clinical profile should be undertaken to rule out the presence of endocrine disorders (E). Thyroid function test (TFT) should be ordered in those with past history of thyroid disorders. Medications for thyroid should be adjusted to ensure that the patient is euthyroid before surgery. In the absence of history, TFT is indicated if there is clinical suspicion of hypothyroidism or presence of goiter (E). If endogenous Cushing's syndrome is suspected, one or more of the following tests should be done: 1-mg overnight dexamethasone suppression test (ONDST), 24-h urinary free cortisol, or 11-pm salivary cortisol (E).

Recommendation 36

Women in reproductive age group scheduled to undergo metabolic surgery should avoid pregnancy. The possibility of improvement in fertility after surgery should be discussed. It is recommended to avoid pregnancy for 12-18 mo following surgery (B). Oral contraceptives or hormone replacement therapy should be discontinued 1 mo before surgery to decrease the risk of thromboembolism (E). If there is clinical suspicion of PCOS, total and bioavailable testosterone and USG of the pelvis assist in establishing the diagnosis. Hypogonadotropic hypogonadism (HH) is commonly reported in males with DM and obesity. Luteinizing hormone, follicle-stimulating hormone, and testosterone total should be measured in males if HH is suspected (E).

Recommendation 37

A decision to evaluate for monogenic or syndromic causes of obesity should be individualized (E).

Discussion

Thyroid disorders: The guideline by AACE/TOS/ASMBS/OMA/ASA recommends that patients known to have hypothyroidism should undergo TFT before surgery, and thyroxine dose should be adjusted to achieve euthyroidism[33]. A meta-analysis of 24 studies demonstrated that metabolic surgery decreased the thyroid stimulating hormone, free triiodothyronine (FT3), and total triiodothyronine levels. Additionally, thyroxine requirement was reduced in overt and subclinical hypothyroidism[238]. Preoperative FT3 above reference range and thyroid autoimmune status in euthyroid persons was shown to correlate with weight loss after metabolic surgery in small studies[239,240]. Larger studies are required to corroborate these findings. There is also a paucity of evidence to support routine preoperative evaluation of thyroid status in patients before surgery, but many insurance providers advocate it. Thyroid profile should be obtained if there are suggestive clinical features or if a goiter is present.

Cushing's syndrome: Cushing's syndrome has rarely been reported in patients undergoing metabolic surgery and should be ruled out by ONDST, 24-h urinary free cortisol, or 11-pm salivary cortisol if there is clinical suspicion[241-245]. If the screening tests are positive further evaluation is required.

Pregnancy and fertility: Most current guidelines recommend avoiding pregnancy if metabolic surgery is scheduled for a period of 12 to 18 mo after surgery[33,223,246]. A meta-analysis of 33 studies analyzing 14880 pregnancies after metabolic surgery indicated that pregnancies after restrictive surgeries tend to have a better perinatal outcome than after malabsorptive procedures[247]. In another meta-analysis, malabsorptive procedures as compared to restrictive procedures, were shown to increase the risk for small-for-gestational-age infants ($P = 0.0466$) but decreased the chance of large-for-gestational-age infants ($P < 0.0001$)[248]. Fertility rates in obese women with infertility were investigated in the meta-analysis by Milone *et al*[249]. Spontaneous pregnancy occurred in 58% of the 589 infertile women after surgery. Women in the reproductive age group should be counseled about the possibility of improvement in fertility after surgery, and contraceptive choices should be considered. The bioavailability of oral contraceptives can be decreased after malabsorptive procedures, and alternative contraception methods should figure in the conversation [250]. Estrogen preparations increase the risk of thromboembolism and should be discontinued 1 mo before surgery[33,246].

PCOS: Three meta-analyses have analyzed PCOS in relation to metabolic surgery[251-253]. PCOS was reported to be present in 36%-45.6% of women before surgery. Resolution of PCOS occurred in the majority of the cases after surgery[251,252].

Male hypogonadism: One of the meta-analysis reported the prevalence of male obesity-associated secondary hypogonadism to be 64%, with resolution occurring in 87% of patients following surgery[252]. A review demonstrated that metabolic surgery was more effective than a low-calorie diet (LCD) in improving free and total testosterone in obesity-associated HH[254]. Additionally, T2DM is also associated with low testosterone levels[255]. In the presence of suggestive clinical features, we recommend to rule out PCOS in females and HH in males during preoperative evaluation.

Monogenic or syndromic obesity: A genetic cause (monogenic or polygenic) is responsible for 5%-10% of early-onset severe obesity[256,257]. Non-syndromic monogenic obesity usually results from the affection of the leptin-melanocortin pathway[258,259]. Syndromic obesity refers to childhood-onset severe obesity associated with dysmorphism and neurodevelopmental and systemic malformations [260]. The common variants are Prader Willi, Bardet–Biedl, Cohen, and Alström syndromes[261]. There is limited evidence to support the role of metabolic surgery in genetic and syndromic obesity at present[256]. A cohort of 133 obese patients with monogenic obesity present among 8.4% of the candidates, were followed up 6 years after LSG. Subjects with monogenic obesity had less short and long-term weight loss than those who did not carry any mutation[262]. Similarly, metabolic surgery was ineffective in causing long-term weight loss in five patients with Prader Willi syndrome over a 10-year period[263]. The evidence to routinely screen for genetic causes in patients undergoing metabolic surgery is inadequate.

PSYCHOLOGICAL ASSESSMENT

Recommendation 38

Patients planned for metabolic surgery should be considered for a behavioral and psychosocial evaluation by a psychiatrist or psychologist with expertise in bariatric patients. Factors that can adversely affect the long-term outcome after metabolic surgery should be addressed (B).

Discussion

The health-related quality of life in severe obesity is worse in comparison to non-obese counterparts[264]. The psychiatric comorbidities in these patients are also high[265]. A formal psychological evaluation is suggested in the current guidelines[33,136,266]. A meta-analysis assessing preoperative mental health in 65363 patients before surgery, reported depression to be present in 19% and binge eating disorder in 17%. These

conditions, however, did not consistently affect weight loss after surgery. On the other hand, moderate-quality evidence demonstrated that the severity and prevalence of depression decreased after surgery[267]. A meta-analysis reported the overall rate of suicide after surgery was 0.3%, which was less than the general population rate of 1.4%[268]. Previous studies have documented a higher risk of suicide, especially in patients with underlying psychiatric disorders[269,270]. The possibility of higher risk of self-harm and suicide attempt after surgery was also suggested in another meta-analysis[271]. The International Federation for the Surgery of Obesity and Metabolic Disorders (IFSO) position statement considers severe and untreated psychiatric conditions like bipolar disorders, schizophrenia, active alcohol, and substance abuse, and bulimia nervosa contraindications for surgery[272]. Preoperative and postoperative psychosocial interventions, especially cognitive behavioral therapy, positively impacted eating behaviors such as binge eating and emotional eating and psychological functioning, including quality of life, depression, and anxiety[273]. The panel suggests psychological assessment to identify underlying comorbidities like depression and eating disorders and rule out alcohol and substance abuse and other frank psychiatric conditions that may interfere with surgical outcome. The patient's perception about contributors to obesity should be discussed, and the ability to cope with lifestyle changes after surgery as well as self-harm tendency should be assessed. Psychosocial interventions such as cognitive behavioral therapy might be beneficial, but structured RCTs analyzing the effect of such a strategy are few.

STRATEGIES FOR PREOPERATIVE WEIGHT LOSS

Recommendation 39

The benefits of preoperative weight loss have not been consistently proven though it has been mandated as a prerequisite by many insurance companies. Lifestyle interventions resulting in weight loss should be encouraged, however evidence demonstrating its benefit is inconsistent (E).

Recommendation 40

More aggressive strategies like very low-calorie diet (VLCD) (450-800 kcal per day) and LCD (800-1200 kcal per day) for 2 wk or more, not only induce weight loss but additionally decrease liver volume and technically assist in performing laparoscopy. There is lack of evidence to routinely recommend weight loss with VLCD and LCD before surgery although it can be considered as per institutional practice (C).

Discussion

Preoperative lifestyle changes lead to a mean weight loss of 7.42 kg in a meta-analysis but there was no effect on mortality or morbidity. The hospital stay was however reduced in the weight-loss group[274]. A meta-analysis suggested that intra-gastric balloon placement and very low-calorie diet (450-800 kcal per day) were the two most effective ways of achieving preoperative weight loss, while another meta-analysis in patients with BMI ≥ 50 kg/m^2 found only LSG and VLCD to be beneficial as bridging interventions[275,276]. Both LCD and VLCD induce weight loss and result in liver volume reduction[277,278]. The meta-analysis by Naseer *et al*[279] included four RCTs using VLCD and four other employing LCD. The authors inferred that the likelihood of achieving 5% weight loss was highest with a three week 700-1050 kcal diet, comprising of moderate carbohydrate, high protein and low or moderate fat. Though both LCD and VLCD cause preoperative weight loss the utility of such a strategy in improving perioperative outcome has not been validated[200]. The recommendation for preoperative weight loss to reduce liver size in order to improve the technical aspects of surgery was downgraded due to inconsistent evidence in the AACE/TOS/ASMBS/OMA/ASA guidelines published in 2019[33].

CONCLUSION

Obesity and DM are complex medical conditions and metabolic surgery is one of the few therapeutic options that alter their tendency for recidivism and progression. Individuals with diabetes are eligible for surgery at lower BMI cut-offs and emerging evidence suggests that the BMI threshold might be further decreased for Asians. Appropriate medical management of these disorders is however a critical prerequisite

for surgery. Our statement provides suggestions for systematically addressing the various conditions associated with DM and obesity that requires optimization before surgery. Dissemination and implementation of these guidelines would help to standardize the management of these comorbidities and improve the perioperative and long-term outcomes. Though these guidelines are comprehensive and up to date, more effort would be needed constantly, to update the rapidly evolving medical literature pertaining to this subject.

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REVIEW

Estrogens and the regulation of glucose metabolism

Marià Alemany

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Marià Alemany, Faculty of Biology, University of Barcelona, Barcelona 08028, Catalonia, Spain

Corresponding author: Marià Alemany, PhD, Emeritus Professor, Faculty of Biology, University of Barcelona, Av. Diagonal, 643, Barcelona 08028, Catalonia, Spain.
malemany@ub.edu

Abstract

The main estrogens: estradiol, estrone, and their acyl-esters have been studied essentially related to their classical estrogenic and pharmacologic functions. However, their main effect in the body is probably the sustained control of core energy metabolism. Estrogen nuclear and membrane receptors show an extraordinary flexibility in the modulation of metabolic responses, and largely explain gender and age differences in energy metabolism: part of these mechanisms is already sufficiently known to justify both. With regard to energy, the estrogen molecular species act essentially through four key functions: (1) Facilitation of insulin secretion and control of glucose availability; (2) Modulation of energy partition, favoring the use of lipid as the main energy substrate when more available than carbohydrates; (3) Functional protection through antioxidant mechanisms; and (4) Central effects (largely through neural modulation) on whole body energy management. Analyzing the different actions of estrone, estradiol and their acyl esters, a tentative classification based on structure/effects has been postulated. Either separately or as a group, estrogens provide a comprehensive explanation that not all their quite diverse actions are related solely to specific molecules. As a group, they constitute a powerful synergic action complex. In consequence, estrogens may be considered wardens of energy homeostasis.

Key Words: Estrogens; Insulin; Estrogen receptors; Energy metabolism; Glucose; Antioxidants; Metabolic syndrome

Core Tip: Estrogens play a paramount and continued regulatory role, based on the synergy between the different forms of estrogen to maintain energy (and lipid/glucose) homeostasis. These functions include preventing: oxidative damage, lipid-induced inflammation, excess fat accrual and the complications of excess amino nitrogen. This short incomplete list is fairly close to a recipe for preventing the development of metabolic syndrome; abundant epidemiological and (partial) experimental data help

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support this assertion. We have to look more widely at estrogens (the different structural-functional types described in the text) to understand their extensive and powerful control of energy homeostasis.

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INTRODUCTION

The complex and growing implication of steroid hormones in homeostasis

Steroid hormones are derived from sterols, which play a critical role in the structure of the (mainly) eukaryotic membrane[1]. Most steroid hormones regulate animal functions, especially in vertebrates. Only recently, plant steroid hormone analogs such as the brassinosterols[2] have been found to play a significant role in the metabolic regulation in the most evolved plants[3,4]. Other steroids, including estrogens have also been found to act as regulators in some plants, but the information is still relatively scarce[5,6]. In addition, a number of higher plants are able to synthesize animal steroid hormones, such as testosterone and estrogens[7], as well as structural analogs which interfere with physiological functions and vital cycles of some animals [8,9], including direct allopathic interference[10]. The steroid hormone ecdysone is critical for molting of insects and other animals[11], and plants synthesize the analogs ecdysteroids to limit insect development[12].

Nevertheless, the most studied types of steroid hormones (estrogen, androgen, corticosteroid, progesterone) are typical and characteristic of vertebrates. However, the variability in functions, regulation and even mechanism of action is considerable, since the degree of implication of these hormones in the fundamental aspects of life: reproduction, feeding-growth-metabolism, neural and metabolic regulation, fall squarely in their fine tuning of life cycles, survival and evolution. Their effects, largely gene expression modulation, are being continuously uncovered, in a way that deviates from the classical distribution of functions for human steroid hormones, often presented as mere sex-definition signals or regulators of mineral and glucose homeostasis. The implication of all of these hormones in the defense mechanisms (including a massive implication in the immune system control[13,14] and optimization of metabolic function[15,16]) has been growing in importance in parallel to their development along the evolution path[17] that brought humans to their amazing homeostatic resilience.

This linear review is focused on estrogens, one of the most important vertebrate steroid hormone types, due to their critical function on the control of core metabolic partition in addition to their fundamental immune system control and reproductive functions.

The estrogens are not only "sex hormones"

Most of the investigations of estrogen effects on metabolic regulation, irrespective of sex, are fairly recent and notably skewed (Box 1). So far we have only limited information on the major role played by different forms of physiological estrogens in the control of energy metabolism at the whole body level[16,18].

The initially intense development of research on estrogens came to a climax by the mid XXth century[19,20], and was essentially focused on their pharmacology, as part of the development of combined estrogen-progestogen preparations for safe birth control in humans[21]. The studies on steroid hormones were not limited to estrogens (and progestogens), but were extended to androgens[22] and, especially, to glucocorticoids [23] through the development of a large number of synthetic drugs, widely used and which their development continues[24]. In a way, this expansion in the pharmacology of steroid hormones also provided considerable information on their mechanisms of action[25] and metabolism[26], including an extensive analysis of some possible complications of their clinical use[27-29]. Nowadays, the natural human corticosteroids (cortisol and cortisone, but also corticosterone[30]) are seldom prescribed, despite showing often quite different effects and pharmacological profile than the

myriad of synthetic corticosteroids in use[31]. The latter may bind to most of the natural receptors[32], but basically do not share the transporter proteins[33] or the inter-organ self-regulatory mechanisms of natural hormones (*e.g.* the hypothalamus-pituitary axis).

The common identification of "estrogen" with 17β-estradiol (E2) and "androgen" with testosterone (T) is an inadequate oversimplification that helps to dismiss the regulatory and fine-tuning interrelationships of the different molecular species of estrogens, both with themselves or with androgens and other steroid hormones.

The estrogens are ancient regulatory agents, remarkably preserved along evolution. The number and structure of relevant molecules remains small and unaltered in spite of the variety and complexity of the mechanisms modulating their actions, somehow reflecting the cumulative experience (and expansion of metabolic interventions) acquired during evolution. The versatility of the nuclear receptors' modulation and signaling pathways allow the superposition of a dense web of signals, including fail-safe, duplicate, alternative and redundant mechanisms, which often make it difficult to find answers to the direct questions relevant to the clinicians.

The structures of estrogens

The principal distinguishing feature of animal estrogens is the phenolic nature of ring A, usually with a –OH in C3. No other type of steroid hormone contains a phenolic ring. The steroid nucleus of estrogens has 18 C, and lack a side chain. The main human functional estrogens are 3-hydroxy-17-keto-estrin (E1 estrone), 17β estradiol or 3, 17β-dihydroxy-estrin (E2 estradiol) and 3, 16α, 17β-trihydroxy-estrin (E3 estriol); during fetal development[34], another estrogen should be included: 3, 15α, 16α, 17β-tetrahydroxy-estrin (E4 estetrol). Compared with all other mammalian steroid hormones, they are highly lipophilic (E1 > E2 > E3). This peculiarity facilitates their transport by lipoproteins[35] and binding to membranes[36] (including their crossing). The interactions with lipophilic entourages have been credited as a main factor for their effects on membranes[37] and mitochondrial function[38]. It is often assumed that estrogens are carried in the blood bound to proteins, largely sex hormone-binding globulin (SHBG), but the higher affinity and metabolic response to energy changes[39] of T (competing with E2 for SHBG) favors a closer dependence of the globulin levels and/or structure/affinity[40,41]. The much lower levels of E2 than T in plasma (both in women and men)[33] suggest that this dual (if real) transport of hormones may be a consequence of modulation of the molecular affinity of SHBG, in part through modification of its molecular weight[39,40,42]. The key factor is that under physiological conditions SHBG (or a varied group of SHBG isoforms) binds essentially T[39] and estrogen (almost 90% of plasma E2, but practically no E1[33]). In addition, the *in vitro* estrogenicity of E1 is considerably lower than that of E2[43]. This fact, together with the abundance of E1 in men (despite being an estrogen), and its high lipophilia resulted in a limited pharmacological interest for this molecule and a consequent lack of literature on it, and its function as a free hormone remains obscure. E1 is the most abundant estrogen in the body (when its esterified forms are included)[44], since it is produced (and stored[44]) in large amounts in white adipose tissue (WAT)[45]. Probably because of its lipophilia, a large portion of E1 in plasma is found esterified as sulfate, much more soluble than the free hormone[46,47], which facilitates its transport and eventual excretion. However, E1 can be made even more lipophilic by esterification with a fatty acid on C3[48] yielding acyl-estrone (AE1). In this form it has been found in lipoproteins[49] and adipose tissue[44]. AE1 are synthesized by adipocytes and modulated by leptin and insulin[50].

E2 is also esterified with fatty acids (acyl-E2 or AE2), becoming more lipophilic than E2, and thus also found in blood lipoproteins[51,52]. However, AE2 is largely esterified in C17 and not in C3 as are the AE1 esters[53]. This peculiarity of AE2 has been attributed to its higher capability to protect the lipids which surround the hormone from oxidation thanks to the unaffected phenolic –OH[51]. In any case, the highly lipophilic estrogens (through esterification with long-chain fatty acids) are a common occurrence for which no definitive function has yet been fully agreed upon, and which shows that the usual molecular species of natural estrogens, their transport in the bloodstream including their binding and physiological functions are far from being fully known. The high concentration of these varied estrogen-acyl-ester molecules in tissues, such as WAT[54] suggests a possible role of reserve or storage of preformed estrogenic molecules[53], which has been explained in part by the easiness of their synthesis by acyl-transferases, widely present in lipoproteins and tissues[55].

Main gender differences in human estrogen function

The scant number of in-depth non-clinical or pharmacological studies may be in part a consequence of the bias against estrogens (and of their bad name, Box 1). The reasons usually presented to sustain negative opinions, (which in the end limit metabolic analyses, and the eventual therapeutic use) are based largely on two factors: their known role as promoters of some forms of cancer, mainly breast[56] and endometrial [57], and a number of risks derived from their use[58,59] other than their assumed role as "female-linked" hormones. The essentiality of estrogens has been proven (in both sexes) for many functions, such as those related with sex differentiation and reproduction, as well as bone health[60]; but the key factor is the increasing flow of data that establishes a direct implication of estrogens in the control of the basic core of energy metabolism[16,61]. This control is affected by age, sex and diet; thus, the simpler division of steroid hormones function using a strict sex-oriented focus is no longer applicable. We simply need to know more about the estrogens and their functions, in exactly the same way as any other hormone, keeping in mind the species-specificity in some of their functions when establishing comparisons with animal models (Box 2).

Women have higher circulating levels of E2 than men, from puberty to menopause, with notable variation between physiological situations[62]. Men, even at their maximal reproductive capacity age, also show fairly high blood levels of E2[63]. There are not enough data on AE2 levels and distribution to establish valid comparisons, but it is probable that the parallelism will be maintained. On the other side, seldom clear gender differences are found in E1, the most abundant estrogen (free or esterified) in human blood. E1-sulfate (SE1) is subjected to a regulative "solubility/excretion" cycle [46] comparable to that of dehydroepiandrosterone[64]. The ample abundance of AE1 in tissues (rat) shows a more marked dependence on the mass of WAT than on sex[65].

T and E2 differently influence brain development from its earliest stages, both in the setting of its functional structure and –later– its psychological orientation and focus[66, 67]. The resilience of women against insulin resistance is higher than that of men[68, 69], at least until menopause[69]. Estrogen protects bone from demineralization in women and men[70,71], a function in part shared by T (at a lower potency, however [72]). The accrual and maintenance of body protein falls largely on androgens, mainly T[73,74], acting in a synergic way with growth hormone[75] and insulin[76] and countering the proteolytic capability of glucocorticoids[77]. The contribution of free estrogens to the maintenance of body protein mass seems to be more limited[78].

Estradiol signaling: classical nuclear receptors. Estrogen receptors α and estrogen receptors β

Thanks to their lipophilic nature, E2 (and E1) can easily cross membranes and bind specific estrogen receptors (ER) within the cell[79]. After dimerization[80] they are brought to the nucleus, where the complex E2-ER binds to deoxyribonucleic acid (DNA)[81] or to specialized proteins[82,83], eventually eliciting the expression or repression of specific genes. The nuclear-type estrogen receptors are highly complex [79,84]. Estrogen signaling, up to its final manifestation is not a fast process such as that of nervous of rapid-signaling chemical regulating agents (Box 3).

Binding to ER is, essentially, specific for the physiological estrogens[75,85], but a wide number of plant secondary metabolism compounds, synthetic non-steroidal estrogenic drugs and even some toxic industrial waste also bind the ER[86]. In humans, there are two main types of nuclear estrogenic receptors: estrogen receptors α (ERα) and estrogen receptors β (ERβ)[87]. In fact, ERα and ERβ are two families of related receptors, which maintain the same overall structure but not their complete sequence, the ERs being adapted, adjusted or changed for best effectiveness, in different tissues or because of changing needs[84,88].

The structures of ERα and ERβ are shown in Figure 1. The main dominions are marked with letters (A to F), and correspond roughly (A/B) to a zinc finger and a binding site, activation function site 1 of the ERs (AF1); C is the place for binding estrogen-response elements (ERE) and then DNA[89]; D is a shorter sequence related to the binding of chaperone proteins and to the process of dimerization; and E/F is the ligand binding domain for estrogens and other factors, AF2[84]. The main ligands are the natural estrogens of mammals (E2, E1, E3 and E4), but some drugs, phytoestrogens [10], metals and diverse chemicals (xenoestrogens) can also bind the receptors[90]. Binding to the AF1 and AF2 may result in synergistic effects[91]. The length and distribution of ER parts may change within each receptor family depending on alternate sequences and splicing[84]. The affinity of ERα: is maximal for E2, followed by E1, and that of ERβ is also E2, followed by E3.

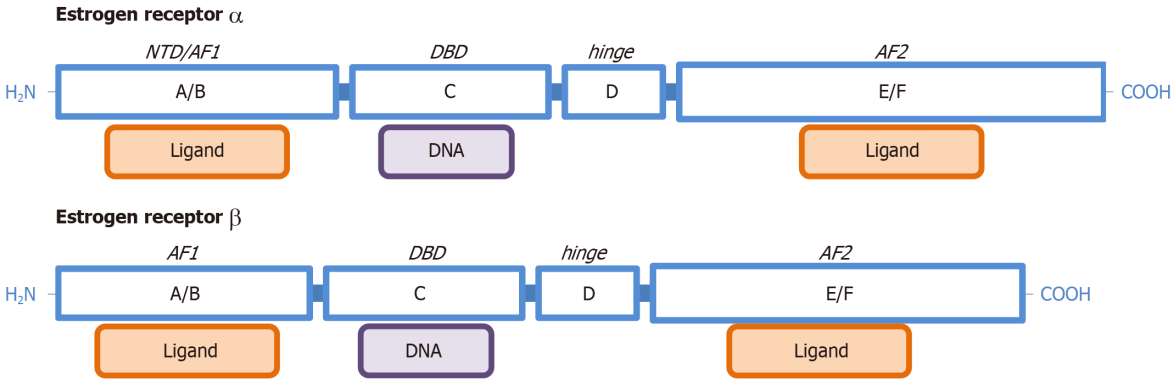

Figure 1 Functional structure of estrogen receptors α and estrogen receptors β. Dominion names (A to F), and common denominations for the main functions are included: N-terminus dominion; DNA binding dominion, activation function site 1 of the estrogen receptors and activation function site 2 of the estrogen receptors. The graph is not drawn to scale and represents the complete (highest molecular weight) form for each of the two families of nuclear ERs. NTD: N-terminus dominion; DBD: DNA binding dominion; AF1: Activation function site 1 of the estrogen receptors; AF2: Activation function site 2 of the estrogen receptors.

The combination of affinities and the panoply of modulators and cell type-specific distribution of ERs results in an extended variety of possible effects, making widely variable the action of estrogens, in order to send specific signals to organs or groups of cells within a wide array of possibilities[92-95]. The ERs are, perhaps one of the best examples of receptor adjustment to the needs of tissues under varying conditions, attained through a considerable number of mechanisms. Both ERα and ERβ are dimeric and coded by different genes[96,97], with an additional abundance of polymorphisms[98,99]. Their distribution in the cells of different tissues and organs is independent for each receptor[100], as are their final gene expression effects[101,102]. They can show synergic[103] or antagonistic[104] effects, even for the same molecular species, and depend, largely, on the post-binding relationships of the E2-ER complex. This situation is further complicated by the interaction of the ER (or E2-ER) with a number of different mechanisms of modulation, such as selective estrogen receptor modulators (SERM)[105-107]; selective ER down-regulators (or degraders)[108]; and the specific ERE, which directly affect the function of ERs[109,110]. Other closely related ("orphan" up to recently) receptors participate in the regulation of critical pathways, but in many cases their relationship with the ERs remains unclear.

Membrane estrogen receptors

In addition to the canonic nuclear ERs, estrogens also bind cell surface ERs[111-113]. As usual with ERs, the terms used to define these receptors shift between the importance given to their location, partial signaling pathway, speed of action and other considerations: membrane ER[114], non-nuclear/non-transcriptional signaling ER[115,116] non-genomic signaling ER; membrane-linked ERα[117,118] or both ERα and ERβ[112]; caveolae- or lipid-related ER[119,120]; G-protein-coupled ER (GPER) [121,122]. This list adds to the existence of alternative or non-genomic "direct" or "rapid" effects of ER stimulation in some cells, eliciting immediate responses (a shift–or development–from the main advantage of the delayed steroid hormone signaling)[123,124].

All these effects suggest that, the ER structure is essentially that described above, with two main types (families) of complete/incomplete ERs: ERα and ERβ, which are found in the cytosol (and nuclei) of cells, including members of the ERα family linked to G-proteins (GPRA1/GPR30)[122,125] attached or close to the plasma membrane.

The proven relationship of ERα to membrane or fat droplet-related structures may be a consequence of the adaptability of ERα[120]; the existence of free and fatty acid-esterified estrogen in lipid-related cell structures[126], or both. In any case, the association of ERα to caveolin-1[119] and then complexed with G-proteins, helps explain the presence-binding of the ER[127], and then E2 in the membrane entourage [128]. In this case, the signal may be transferred to membrane-related structures, as is the case with increasing calcium release[115]. The G protein-ER complex, upon the binding of E2 may also induce nuclear effects *via* activation of tyrosine kinases[129] and the MAP/ERK or PI3α/Akt pathways[130]. The stimulated G protein-activated receptor may also signal through GPR30[131]. The activated system containing the ER also enhances adenylate cyclase activity[132] *via* phosphorylation of the cAMP response element[133]. However, these direct membrane-related mechanisms may

coexist with also faster direct translational actions of conventional ERs somewhat linked to membranes[134] or with other mechanisms hinted at but not fully disclosed yet[135,136]. This includes the presence of ER receptors within cell structures, such as mitochondria (*e.g.* ERβ)[137].

It has been established that non-genomic effects of ER bound to E2 may belong to two confluent mechanism types: direct effects elicited from the membrane and effects developed through cytosol signaling cascades and actions. Both processes are probably coincident for different cell settings. Hypothalamic inhibition of guanylate cyclase[138] and LH secretion[139], as well as increased cell migration[140] and other brain effects[141] add to the widening array of non-genomic effects of ERs. Most of these effects have lately been attributed to the ERα irrespective of the place of binding with E2, but the implication of ERβ has also been described[142,143].

Notwithstanding, all these receptor-related mechanisms described cannot fully explain all the biochemical effects induced by estrogen signaling[144-148], leaving ample space for the assumption of direct, *i.e.* non ER-related, involvement (largely of E2 and its C17-fatty esters, AE2)[149,150]. However, these effects have been described only in lipoproteins, other lipid masses or lipid/protein interfaces[54,151]. The direct effects of estrogens on mitochondria have been related to specific mitochondria receptors[152,153], apparently containing ERα, ERβ[154] and other possible binding structures[155]. Their role on mitochondrial function, however, has been found to be significant[154,156], especially in the regulation of energy providing pathways[157, 158]. The possibility of estrogen direct incrustation in the lipid layer of membranes has been proposed as a way to modify their functionality[159] and enhance the E2/AE2 anti-oxidative properties in a way similar to its postulated function in lipoproteins[160, 161].

Estrone and AE1

E1 is a rather peculiar and resilient hormone (Box 4); we do not yet have a direct explanation for its massive synthesis and storage, since the lipophilic nature of E1 (but not that of SE1) limits its action in plasma, cell and interstitial space. Non-esterified E1 levels are related to those of E2, with E2/E1 ratios fairly stable for men (*c.* 1) and more variable for women (*c.* 1.5-2) up to menopause[162]. However, measurement of circulating estrogen is difficult, often showing poor correlations between instrumental and immunoassay results[163]. The relationship of E1 with E2 levels, in addition to sex (and age) is affected by diabetes/obesity[164]. Furthermore, analyses of SE1 seldom include other E1 esters nor free E1, which compartmentation (important in lipoproteins) skews E1 serum levels towards lower values. The obese show high plasma SE1 concentrations[165]. In any case, the whole-body AE1 content in rats is several orders of magnitude higher than free (and sulfate-esterified) E2[44], however, the AE1 content in obese rats is relatively lower than in normal-weight animals, despite AE1 being essentially stored in WAT[44].

The oral pharmacological administration of oleoyl-E1 to normal weight and obese rats[166,167], induces a marked decrease in fat depots[168], not dependent on the degree of obesity and diet[167,169]. The loss of fat runs parallel to the normalization of glycemia, blood lipids and other metabolic syndrome (MS) markers[170], without apparent effects of estrogenization, and irrespective of energy intake manipulation [171]. AE1 has been proposed as a ponderostat signal[170], since the excess fat is shed without accompanying metabolic disorders[170,172]. Its negative effects on humans are negligible (clinical studies, phase I, unpublished data), and the positive (*i.e.* loss of excess fat, lowered insulin resistance, absence of estrogenization) were outstanding in a single case published[172]. However, its development as a drug was abandoned because an ill-designed phase II failed to be conclusive. We have no hints as to the mechanism of AE1 signaling, other than it is synthesized in cultured adipocytes[50], and WAT stores these esters in large amounts[44,65]. AE1 treatment reduces the size of WAT lipid depots[173,174]. Natural AE1 is transported in the lipid fraction (lipoproteins) of blood[49,175]. Methodology is a critical factor for the analysis and tracing of acyl-estrogens, with disparate results; *i.e.*, it has been reported that human plasma does not contain AE1 at all[176].

The main effects of AE1 are a consequence of the structural change on the whole ester, not through the release of E1[177]. When injected, marked estrogenic effects are observed, with increased E1 and E2 levels[178]. However, oral administration of AE1 does not elicit the same signs of estrogenization[171]. A highly critical analysis of oleoyl-E1 actions on rat body weight found no significant negative effects[179]. Body protein and N balance are preserved in AE1-treated (lean and obese) rats[166,167,174, 180]. There is very little information available on AE1 mechanism of action. The structure of the orally administered ester seems to be modified, with low levels in

blood plasma[49,181,182], but an unidentified derivative is present in large concentrations, maintaining the estrogen nucleus in a more hydrophilic form[181]. In liver, AE1 label can be found linked to DNA shortly after administration[181]; the effects of AE1 imply the stimulation of ERα[183,184]. Excess AE1 is essentially excreted as SE1 [185].

There are sufficient elements to sustain the implication of AE1 in the regulation of body weight[170], but the lack of further complete studies on its mechanism of action has prevented both its clarification and its eventual therapeutic application. No other explanation has been put forward to justify the limited estrogenic potency of E1, despite its massive synthesis in the ovary and the brain[186], and, especially (in quantitative terms) in WAT[187], with a direct relationship of its total body content and circulating levels with WAT, lowered by obesity in rodents and humans[47]. The effect of the administration of free E1 to rats induce some estrogenic effects and slightly increases body weight, effects quite different to those of its acyl derivative [177].

Estrogens and the regulation of energy metabolism

Glucose is the main energy substrate, and the main simple nutrient of human diet. Glucose is also the primary inter-organ energy substrate carried by the blood to sustain the energy needs of body cells. Carbohydrates capable of yielding glucose (or other interconvertible hexoses) are a necessary part of our diet[188,189], and for many thousands of years they have constituted the main staple of our energy intake. This role has been already addressed in depth in a previous paper[189] in which we discussed the final fate of dietary carbohydrate, protein and lipids to yield two-and three-carbon metabolites (2C, 3C) and anaplerotic four-and five-carbon (4C, 5C) molecules from proteins (when excess N could be disposed of). The common shared groups of metabolites from dietary nutrients include 2C fragments (and a smaller amount of 3C from glycerol) from fats and, essentially 3C fragments from the six-carbon (6C) hexoses. The 3C could be largely used to maintain glycemia thanks to hepatic[190], renal[191] and intestinal[192] gluconeogenesis, or simply used (pyruvate) as a source of 2C (to yield acetyl-CoA), which is largely oxidized to CO_2 in the mitochondria through the Krebs cycle. Most of the energy drawn from glucose is obtained from the pyruvate-lactate produced in the glycolytic pathway followed by the complete oxidation of pyruvate, as acetyl-CoA, in the Krebs cycle. The 3C fragments (essentially lactate, pyruvate, glycerol, alanine and serine) can substitute glucose as an energy substrate in many tissues, avoiding the strict control of glucose levels, and providing faster access to their energy when and if enough oxidative capability and oxygen are available[193,194]. Glucose isoforms often delay somehow the oxidation of glucose[195], and thus, the direct cell use of glucose-derived 3C fragments may speed up its catabolism. This C6→C3 massive conversion is one of the most important albeit less publicized functions of WAT[196]. The presence of excess lipids (and energy) in the diet often results in an excess of 2C fragments (mainly the result of catabolic oxidation of their polymers: fatty acids) that their oxidation becomes problematic, thus the excess of energy available facilitates their storage (often long term) as fats[189].

The inadequacy of diet composition, and especially the excess of energy from fats and carbohydrate results in the progressive metabolic disorders of MS[197] with the development of sustained hyperglycemia[198], often deriving into type 2 diabetes [199], obesity[200], altered blood lipids, with hyperlipidemia[201], deriving in endothelial inflammation[202,203] and increased cardiovascular risk[204], hepatic steatosis[205], depression[206], and increasingly functional alteration of the nervous system[207], bone[208] and practically all organ/cell systems, extended even to the microbiota[197]; and, essentially, the immune system[209,210]. The causes and effects of MS have been intensively and extensively studied, and a direct relationship has been found with diet composition and excess energy[211,212], but no effective solutions have been put forward. Medical treatment is commonly limited to increased energy expenditure and changes in type of food, and (decreased) energy intake[213-215], in most of the cases, without sufficient metabolic analyses[216]. This is complemented by the pharmacological treatment of the disorders included in the MS. The relative acceleration of the MS effects with age is more clearly observed in adult (and aging) men than in women[217,218]. This difference has been attributed to the obvious diet-driven inflammation of MS[219,220], compounded in men by the progressive decrease in the synthesis (and effects) of T, in part a consequence of aging but also by the hypoandrogenism that characterizes MS[221]. Women, from adolescence to the beginning of menopause maintain their high levels of E2 and functional hypothalamus-hypophysis-gonadal axis[62]. Menopause, aging and other causes

break this equilibrium and the levels and protective effect of estrogens wane; The E1 *vs* E2 ratio of concentrations is maintained at E2 > E1 in premenopausal adult women, changing to E1 > E2 in post-menopausal women and in men (in which there is little change with age). In both cases, E2 levels were lower in men and post-menopausal women than in adult premenopausal women[62].

In aging men, especially those with MS, treatment with T reduces to some extent cardiovascular risk[222,223] and helps maintain glycemia[224,225], but possible dangers, insufficient knowledge and scant physiological analysis have limited the extension of this therapeutic avenue[226]. Similarly, for women, substitutive estrogenization is partly effective[227-229] at menopause, but its extension has been seriously limited by the fear of possible negative consequences, as discussed in Box 1. In addition, synthetic estrogens are the most used substitute drugs despite our very limited knowledge[230] of the intricacies of their action in such complex mechanisms as those described above for E2. The case of tamoxifen (agonist/antagonist) is a clear example[231]. This generalized (albeit undeclared) ban on sex hormones extends to the use of T in women, despite the fact that both E2 and T are needed for bone[70,232] health, and T for body protein maintenance[75]. Obviously there are problems to solve, but it seems that this line of study has not been sufficiently developed for reasons not based on contrasted arguments. In the case of AE1, a line of research developed by only one research group, obtained better results than those of any previous anti-obesity drug[170,179], but the development was discontinued largely for fear of "possible" future negative findings[179].

Estrogens, insulin and dietary nutrients handling

Most of the studies on the effects of estrogen on glucose metabolism have been done using E2 (and other ER ligands). There is a very limited amount of specific information on E1 direct effects; however, SE1 was found to induce hypoglycemia in genetically obese mice *via* glucose-6-phosphatase[233]. The effects of estrogens on glucose and energy handling are mediated through four coordinated actions: (1) Protection and facilitation of insulin secretion and function in the control of glucose availability to tissues; (2) Modulation of energy partition, favoring the use of lipid as the main energy substrate when their availability is higher than that of carbohydrates; (3) Functional protection through antioxidant mechanisms; and (4) Central effects on whole body energy metabolism and homeostasis maintenance.

Estrogens, insulin and glucose

E2 protects the functionality of the pancreatic β cells[234,235], preventing apoptosis [236], adapting their function to insulin resistance[237], and maintaining their insulin content[238]. ER stimulation inhibits lipogenesis in the β cells[158], which limits the negative effects of excess lipid in the cell. The loss of the ER (nuclear and/or membrane) impairs pancreatic insulin secretion[239], which is stimulated by estrogenic signaling[240]. The lack of E2 availability also increases hepatic insulin clearance[241].

Estrogens also prevent the development of diet-induced insulin resistance[242]. The gender-dependent effects of estrogen on high-fat diet-induced insulin resistance are largely dependent on the anti-inflammatory effects of the hormone[243]. E2 increases tissue insulin sensitivity[244], and lowers insulin resistance in peripheral tissues[245], with marked differences in the effects depending on gender[243]. In female mouse adipocytes, E2 lowers inflammation (and thus insulin resistance[246]), and enhances the effects of insulin on tissues[247]. The sole activation of ERα AF-1 is enough to prevent obesity, liver steatosis and insulin resistance in mice[248]. However, obesity and insulin resistance seems to require E2 in addition to ERα and AF-2, AF1 not being essential[249].

Estrogens induce a considerable number of actions in the brain, which is also able to synthesize them[250], playing an important role in its function[251,252] and behavior [253]. E2 also interacts with serotonin to affect insulin resistance[254]. Estrogenic deprivation induces mitochondrial dysfunctions in the brain which may induce the loss of cognitive functions[255]. More complex is the long saga of the relationship of estrogen in the peculiar placing of brain insulin resistance in Alzheimer's disease[256, 257]. The neuroprotective actions of estrogens[258], added to the inhibition by E2 of β-amyloid production[259] and the implication of E2 in the regulation of insulin degradation in the brain[260], suggest an overall beneficial effect of estrogen limiting the development of this disease. However, Alzheimer's disease affects more women than men[261], and a number of caveats have been raised against the danger of natural estrogens being implicated in its development[262]. Right now the case is not solved, with studies showing a protective effect of ERβ[263] and others hinting at the

implication of ERα in its pathology[264].

Estrogens, largely E2, facilitate the uptake of glucose from the intestine[265], and its extraction from the bloodstream by activation of transporters GLUT4[266] and, at least in the brain[267] GLUT1. E2 lowers liver glucose output with no changes in glycogen during mild exercise[268], a difference due in part to a modulable maintenance/inhibition of gluconeogenesis[269]. E2 also regulates glycolysis in endothelial cells by non-genomic pathways[270], partly by increasing insulin signaling[271]. Glucose catabolism is affected by estrogens, which stimulate glycolysis *via* phosphofructokinase[272], and the pentose phosphate pathway *via* Akt[273]. In any case, the direct incidence of ER signaling on glucose handling is relatively limited and conducted *via* modulation of insulin[271]. Probably, the main effect of estrogen may be the utilization of lipids as alternative energy substrates. This is important for humans, because of the common occurrence of excess lipids (and energy) in Westernized diets, which leads to problems in dietary substrate partition[189] and the common development of MS.

Lipid handling and estrogens

Estradiol: Estrogens lower circulating triacylglycerols (TAG) favoring their transport with a higher expression of ApoA5[274], and protecting lipoproteins against oxidation [275]. However, the main effect of E2 on lipids is favoring the shift from lipid deposition (storage) to its oxidation as energy substrate. Perhaps this is the most critical effect of estrogens on energy partition.

Treatment with E2 decreases obesity[276], protects against hepatic steatosis[277], lowers the activity of cholesterol acyl-transferase[278] and limits fat deposition[279]. All these are –again– indirect actions aimed to decrease the storage of excess TAG, since E2 does not directly regulate lipolysis[280]. Nevertheless, estrogens decrease lipogenesis[281] in WAT; and adipogenesis is also inhibited through ERα activation [282].

Dietary composition directly affects the substrate partition and the regulation of substrate utilization to maintain both energy and nutrients homeostasis[283]; in rats, hyperlipidic diets induce increases in E2 levels, and are correlated with an increased use of fatty acids as energy substrate[284]. The decrease in lipogenesis/adipogenesis (and the relatively enhanced lipolysis) frees the use of excess glucose and glycolytic 3C towards 2C and its oxidation in mitochondria; thus, decreasing the synthesis and storage of fatty acids (and TAG).

The effects of E2 on lipid handling are coordinated with the actions of E2 on insulin [235,285], glycemia[286] and the use of glucose as the direct energy substrate[271] instead of using it to fuel the synthesis of fatty acids. E2 lowers insulin resistance and fat storage through the ERα and the FA2 binding site[249]. Estrogens also lower the insulin resistance induced by excess dietary lipids[245].

A key point of these E2-derived metabolic shifts lies on the mitochondria[287,288]. Estrogen controls mitochondrial biogenesis and function[289]. E2 deprivation induce mitochondrial dysfunction and insulin resistance, which may induce alterations in the cognitive ability of subjects[255]. E2 potentiates the oxidative capacity of mitochondria, through increases in cAMP and cytochrome C oxidase activity[290]. E2 also inhibits the synthesis of adenosine triphosphate (ATP) in the mitochondria[291], which may be related to the increase in oxygen consumption and energy expenditure elicited by E2 [292], and its postulated role enhancing heat production and thermogenesis[293,294], which imply a higher overall substrate oxidative activity.

The pyruvate dehydrogenase complex (PDH) is a critical control node, which catalyzes the irreversible conversion of 3C pyruvate to 2C acetyl-CoA in the mitochondrion. The main mechanism of PDH control is phosphorylation, mainly by (inhibiting) PDH kinase 4 (PDHK4)[295]. Insulin inhibits the expression of PDHK4 [296], which has an increased activity during starvation and diabetes[297]. The levels of PPARγ coactivator-1 α (PGC-1), an important cell energy regulator[298,299], which is also increased during diabetes and starvation, modulates the function of ERs[300]. PGC-1 increases hepatic gluconeogenesis through the expression of phosphoenolpyruvate-carboxykinase[301], and co-activates, with estrogen-related receptor (ERR), the expression of glucokinase[302]. PGC-1 increases the expression of PDHK4 [303], essentially through the activation of ERR (mainly the ERRα and ERRγ isoforms) [304]. ERRs are homologous to the nuclear ERs, but they are orphan receptors, *i.e.* do not have specific ligands such as E2[305]. Recently, it has been suggested that PGC-1 could be considered, perhaps, their unique non-steroid ligand[306]. ERRs increase glycolysis and glucose uptake[307].

The activation of PDHK4 by ERRs and PGC-1 is inhibited by insulin[303]. However, E2 activates ERRs[308]. In sum: insulin activates PDH, which is inhibited by ERRs modulated by cell lipid energy conditions (PCG-1) in a way that facilitates a decrease

in insulin resistance[309] and a steady flow of 3C to 2C into the mitochondria to fuel the Krebs cycle, since lipogenesis is inhibited[281,310] and cannot absorb the newly formed acetyl-CoA.

Further stimulation of mitochondrial oxidative capacity[153,311], and the availability of 4C and 5C derived from amino acids, further speeds up the oxidation of 2C by the mitochondria of liver, WAT and specific brain sites[312]. The accessibility of amino acid hydrocarbon skeletons depends on their increased oxidation (when in excess and limited capacity of the Krebs cycle[189]) *via* the alternative oxidation of amino groups to nitrogen gas[222,313]. The presence of these anaplerotic fragments and the higher oxidative capacity markedly increase the use of acetyl-CoA (from fatty acids or glucose) as the main energy substrate. The added relative inefficiency in the production of ATP[291] further helps the estrogen-controlled metabolism of adult women to dispose (albeit partially) of unwanted excess dietary energy. This effect may account in part for the resistance of women to develop the MS in its double facet of obesity and diabetes[314].

Estrone and acyl-estrone

E1 has not generated as much literature as E2, but this is probably a consequence of its limited direct effects on classical estrogenicity and energy metabolism. However, it has been found that SE1 also contributes to glucose homeostasis, inhibiting glucose-6 phosphatase under conditions of hyperglycemia[233]. SE1 also lowers the levels of lipoproteins in postmenopausal women[315]. And, obviously shows estrogenic effects when given in pharmacological doses, albeit less marked than those of E2.

The anti-obesity effects of oleoyl-estrone, an AE1 ester, were studied extensively for a short time[170], but ceased before the appearance of many key studies on estrogen function and mechanism of action cited above. Thus, these older studies have to be re-analyzed from the present-day perspective. The acyl moiety of AE1 comparatively affects only partially its slimming effects[48], thus, oleic acid (the most abundant in the rat body stores) was used as standard. The E1 moiety, surprisingly, is not essential either, since both AE2 (at pharmacological levels) and oleoyl-diethyl-stilbestrol show marked body fat slimming effects[48]; however, these compounds have not been studied further because of the marked estrogenic response (toxic at the pharmacological levels analyzed) they elicited in comparison with AE1 or even E1 alone.

AE1 is not estrogenic[171]. However, the injection of liposomes loaded with AE1 induces estrogenic effects in rats due to the large amount of E1 produced by its hydrolysis[178]. The oral administration of AE1 basically excludes most of the E1[316] formed in the intestinal hydrolysis of AE1. In any case, SE1 is, finally, the main catabolite of AE1[185] (and of E1), since the fatty moiety poses no problems to its complete oxidation. This way, the interference of estrogenic effects has been circumvented simply by oral (instead of *i.v.*) administration of AE1, allowing a more direct analysis of its effects on body lipids[167,169,173]. The administration of AE1 preserves body protein[167,169,317], but markedly decreases body fat through the maintenance of a negative energy balance[167,169,177,180,317]. The process is achieved by lower food intake and unchanged thermogenesis[180] (an effect partly shared by E2 but not by E1), as well as a shift in the management of dietary fat, from accrual to oxidation for energy[88,318], such as described for E2. The effects of additional reduction of food availability are comparable (and additive) to those due to decreased appetite elicited by AE1[317]. Circulating levels of AE1 are proportional to body fat[65,182]. However, obese rats have lower AE1 levels than their lean counterparts[44]. In sum, the main effect of AE1 (given at pharmacological levels) is to shed excess body fat, without additional metabolic interference[174]. AE1 circulating levels presumably act as an indicator of whole body fat reserves under normal (not MS) conditions[319]. The AE1-induced loss of body TAG implies the concordance (described for E2) of peripheral (especially WAT) lipolysis[320], decreased lipogenesis[321] and higher energy expenditure and lipid oxidation[318].

The effects of AE1 on glucose metabolism are comparable to those described for E2: regularize hyperglycemia[174], decrease insulin resistance[174] and an overall antidiabetic action[322]. However, these effects may be just the consequence of the normalization of energy homeostasis induced by pharmacological doses of AE1[170], with full activation of the estrogen shift explained above: increased mitochondrial oxidation of 2C (and excess 3C) instead of storage of excess 2C mainly in the form of TAG-fatty acids.

The similarities of AE1 with E2 are both quantitative and qualitative. The well-known summarization of E2 function as less estrogenic than neuroprotective[323] is not applicable to the comparison with AE1 because these esters do not show either of these functions. Nevertheless, injected AE1 label has been found in cell nuclei[181],

and AE1 binds the ERα[183,184], but E2 cannot displace AE1 from its binding[171]. In addition, the pharmacological effects of E2 and AE1 are not superimposable[177]. This is compounded by the lack of full inhibition of AE1 actions on rodents by tamoxifen [324] and fulvestrant[184] (in fact, tamoxifen mimicked some of the effects of AE1[324]). These data help finally differentiate the effects of AE1 from both E1 and E2, and suggest that AE1 is, probably a SERM.

Functional protection through antioxidant mechanisms

E2 and E3 (but not E1) are considered effective antioxidants[325], since they help protect structural lipids from free radicals[326]. The polarized structure of estrogens makes them ideally suited to interact in interfaces between hydrophilic and lipophilic media[327], such as membranes, including mitochondria[160]. In this aspect, perhaps the AE2 esters may be the most effective, because in addition to E2, their most common acyl moiety, linoleic acid[328], is itself a main component of membranes [329], albeit being easily oxidized by free radicals[330]. The AE2 have been described as powerful antioxidants, more effective than free E2[331]. This role includes mitochondria, closely related to estrogen action for increased numbers, oxidative capacity, metabolic function and survival[161]. In this sense, both E2 and AE2 (and, probably to a lesser extent E1), control[332] and protect mitochondria in brain, liver and other tissues[156,161,333]. The antioxidant effects of estrogens seem unrelated to the classical estrogenic activity[334].

The AE2 antioxidant function is not limited to membranes, since their presence in lipoproteins helps protect them from scavenger radicals[275], maintaining their transport and signaling function. Since acylation on C17 of E2 results in a more effective antioxidant molecular type[55], and no other estrogens seem to specifically carry out this task, its uniqueness, and the importance of the function suggests that the AE2 may constitute, by themselves, a different specialized type of estrogens carrying out a critical and specific function for which they are best suited.

Whilst AE1 do not show significant estrogenicity[171], AE2 are markedly estrogenic [126,150,244], and maintain this estrogenicity longer than the 3-acyl-E2 esters[150], which suggests that they may–precisely-retain this property when packed in lipoproteins, such as low density lipoprotein (LDL)[126] or bound to plasma proteins [335]. When taken together, these properties suggest that the AE2 may, at least, fulfill the role of transport/storage of E2 in addition to an antioxidant function.

Central-mediated effects of estrogens on energy homeostasis

The main arguments for the postulated subdivision of estrogens in four separate classes, based on their structure and function is based on widely different availability of sources, but the marked differences observed suggest -at least- the existence of four groups, described in Table 1, which summarize most of the information provided in the present study.

E1:

Structural: Estrin nucleus, with only one phenolic-OH.

Functional: Mild estrogenic effect; a main precursor in the synthesis of E2; main catabolite in the excretion of estrogens as SE1; SE1 being probably the main signaling form of E1; increases growth during development; quantitatively the most abundant molecule with an estrogen nucleus in the body; possible "reserve" for rapid conversion to E2 or AE1.

Targets: (Generalized); WAT; reproductive-system organs.

AE1:

Structural: Estrin nucleus, with only the phenolic-OH esterified by a fatty acid.

Functional: No estrogenic effects; product of esterification (or interchange) of E1, probable active SERM for ERα, activates lipid catabolism, *via* lipolysis and oxidation of fatty acids; postulated as ponderostat signal, markedly lowers body fat: maintains glycemia.

Targets: WAT; brain; liver.

E2:

Structural: Estrin nucleus, with the phenolic–OH, and another-OH in C17.

Functional: Main estrogen; marked classical estrogenic effects, protects insulin and facilitates its secretion, maintains glycemia; indirectly activates lipolysis and inhibits lipogenesis; protects and favors the increase and oxidative function of mitochondria, lowers body fat, has antioxidant capability.

Targets: Brain, liver, mitochondria, reproductive-system organs, bone.

AE2:

Table 1 Comparison of the effects/functions between the main functional types of estrogens[1]

Effect/ function/ action/ characteristic	E1	AE1	E2	AE2
Bind the ERs at the hormone binding site	↑↑[2]	X	↑↑[2]	~
Bind the AF1 or AF2 sites of the ERs	X[2]	↑	X[2]	~
Bind to mitochondria (and some membranes)	~	~	↑[2]	↑[2]
Elicit a direct classic estrogenic response	↑[2]	X	↑↑[2]	↑↑[2]
Induces hypoglycemic effects	~i	↑↑[2]	↑[2]	~
Is carried by lipoproteins	↑[2]	↑↑[2]	X	↑[2]
Show anti-oxidative effects	~	~	↑[2]	↑↑[2]
Activate the 3C→2C conversion (pyruvate dehydrogenase)	~	↑2	↑↑[2]	~
Increase mitochondrial oxidative activity	~	~	↑	~
Increase whole body thermogenesis	~	↑[2]	↑[2]	~
Show lipolytic effects	X	↑	↔	~
Show lipogenic effects	↑	↓[2]	↓[2]	~
Decrease WAT fat mass/ limits fat deposition	↓	↑↑[2]	↑[2]	~
Allow the activation of the alternative N disposal pathway	~	~	↑	~
Decrease body protein mass	↔	X[2]	X[2]	~

[1]Specific early development and pregnancy-related estrogen molecular species and functions not included.
[2]Shows a coincidence of effect/function for different estrogen types in the same row.
↑: Exerts the effect described; ↓: Exerts an effect opposite to that described; ↔: Variable/not univocal responses; X: Does not exert the effect described; ~: Absent or insufficient information available; E1: Estrone; AE1: Acyl-estrone; E2: 17β-estradiol; AE2: Acyl-E2; ERs: Estrogen receptors; AF1: Activation function site 1 of the estrogen receptors; AF2: Activation function site 2 of the estrogen receptors; WAT: White adipose tissue.

Structural: Estrin nucleus, with only the phenolic–OH, esterified in C17 by a fatty acid, often polyunsaturated.

Functional: The most effective estrogen form of antioxidant; postulated as an element of transport or reserve of E2, protects lipoproteins, membranes and cell components, marked estrogenic action.

Targets: Mitochondria; plasma lipoproteins.

This partial (and incomplete) classification of the main estrogens is based on both structural and functional aspects. The estimated quantitative mass of these four types of estrogen (under standard conditions) in the whole body is: AE1 > E1 > E2 > AE2.

Estrogen is a fundamental modulator of female functions (including estrogenicity *stricto sensu*), which agrees with the high levels of E2 in adult pre-menopausal women. No sufficient data are available to show other gender differences in the postulated groups of estrogens, except for AE1, which pharmacological effects in obese rats are more intense in males than in females.

Estrogens, essentially E2, are responsible for the development of the sex-dependent structures (both physiological and psychological) related with mating and reproduction. Evidently this is achieved with the collaboration of other hormones, *e.g.* androgens, progestogens and peptidic hormones. It is unclear whether the other three groups of estrogens depicted in Table 1 play a specific significant role in the reproductive processes (with the exception, perhaps, of AE2).

The other key places for action of estrogens are characterized by containing ERs and are found throughout the whole body[336]; but WAT[337], liver[338], muscle[339], and, essentially the brain[340,341] are the main targets for their actions, and are, at least, the best studied. ERβ globally regulates lipid homeostasis[145], its activation in obesity increases whole body metabolism and mitochondrial biogenesis as a counter-measure to excess WAT lipid storage[312]. Oophorectomy alters WAT lipid metabolism[342], the plasma levels of E2 are affected by diet[284] and determine body fat deposition[343], probably through central (brain) control mechanisms.

In the hypothalamus, where E2 interaction with serotonin has been described[254]. E2 regulates sympathetic nervous control[344]. E2 and AE1 have marked anorectic

effects[180,345]. The postulated ponderostat effect of AE1[181,319] depends on its action on brain, where blood-injected label has been found[181]. The effects of estrogens on glucose and body fat have been attributed to central actions on the brain [15,346]. This is a logical assumption, including also T[347], because the brain controls the body energy metabolism[16] and homeostasis[348], *i.e.* regulates the coordinated biological maintenance systems of the body[349,350].

General considerations and conclusions

The growing number of known actions of estrogens in metabolic control cannot be fully explained by only the analysis of the "common" estrogens, essentially E1, E2 and E3 (with their sulfates). Thus, acyl-estrogen derivatives have also been included: Despite being known for a long time, and used in pharmacology, they are seldom included in general analyses of estrogens. These compounds are quite diverse. However, in practice, the studies available are limited only to acyl esters of E1 (on C3) or E2 (largely on C17); their properties are quite different, starting with estrogenicity, and continuing on to antioxidant or lipid wasting effects (Table 1).

In any case, the differences in effects induced by E1 and E2 (those of E3 seem to be closer to E2), are considerable, both in their classical estrogenic power and in their implication in regulative mechanisms: E2 being more powerful than E1 in almost any aspect related to metabolic regulation, becoming the most representative estrogen. Nevertheless, a large proportion of E2 is synthesized from E1 by widely distributed 17 OH-steroid dehydrogenases[351]. The similarities between E1 and AE1 actions are small, the latter resembling more E2 in its metabolic effects, than E2 *vs* AE2, despite the strong relationships in the main functions of AE2: antioxidant and estrogenic.

Due to the crossed coincidences of effects between E1-E2 and their acyl esters, a loose classification based on functions and structure has been developed and presented here. Either separately or as a conjoint "estrogens" block they may provide a comprehensive explanation of most of the actions of estrogens, which could not be attributed in any way solely to either one or to all the usual non-esterified estrogens as a group. It is also remarkable that these four groups constitute, together, an extensive fully synergic unit of action: antioxidant effects protect mitochondria, membranes and lipids, which are actively used for energy, limiting insulin resistance. However, the protection extends to insulin (and the pancreatic β cells); insulin secretion is maintained to sustain a steady glycemic response. Glucose is converted to 2C only when it is in excess, whilst dietary lipids (at the root of inflammation and development of MS) are not accrued but oxidized. Protein is preserved by strong, effective and well established mechanisms; but excess energy (and glucose) does not prevent the utilization of amino acids for energy, and, especially for efficient operation of the Krebs cycle thanks to the supply of 4C and 5C fragments. The problem of excess N disposal[189], strictly overprotected *via* the urea cycle is compensated using the direct pathway to produce N_2[189]. Lipoproteins remain functional thanks to the steroidal antioxidants, TAG transport is practically unaltered, but lipogenesis is maintained low, and lipolysis high to dispose of excess 2C energy. Thermogenesis is maintained and appetite (and food intake) are adjusted to the real needs of energy intake (plus the use of unnecessary fat reserves). A happy metabolic Arcadia under the rule of essentially one (multiple) hormonal factor: estrogens.

Figure 2 shows the main interactions of simple estrogens (essentially E2) and AE1 on the core of intermediate energy metabolism, the meeting point of carbohydrate, lipid and protein catabolism. The antioxidant effects of AE2 (and E2) have been omitted, since their main point of action lies, just from the critical step 3C→2C and the Krebs cycle, within the mitochondrion. The coordinated implication of estrogen types in the control of this segment of substrate handling is pervasive, both by direct implication and through its modulation of insulin action, and helps clarify that the implication of estrogens (as a whole) on glucose handling (and insulin control) is very high. The control node of energy metabolism lies in the mitochondria/cytoplasm interface, and-perhaps-critically on PDH. Around this point, directly linked to oxidative function of mitochondria and their generation of ATP, the implication of estrogens is high, as are the adjustment of the supply lines of 2C and 3C, the control of glycemia and the shift of amino acid catabolism under conditions of abundant energy (and glucose) availability[189].

In addition to the need to consider the estrogens as a group of several molecular species sharing a common biochemical structure and origin, implied web-like in a large number of coordinated metabolic functions, the main conclusion of this review is, precisely, the paramount metabolic importance of the estrogens. This is based on the synergy between the different forms of estrogen to maintain energy (including, obviously, glucose homeostasis), whilst preventing oxidative damage, lipid-induced

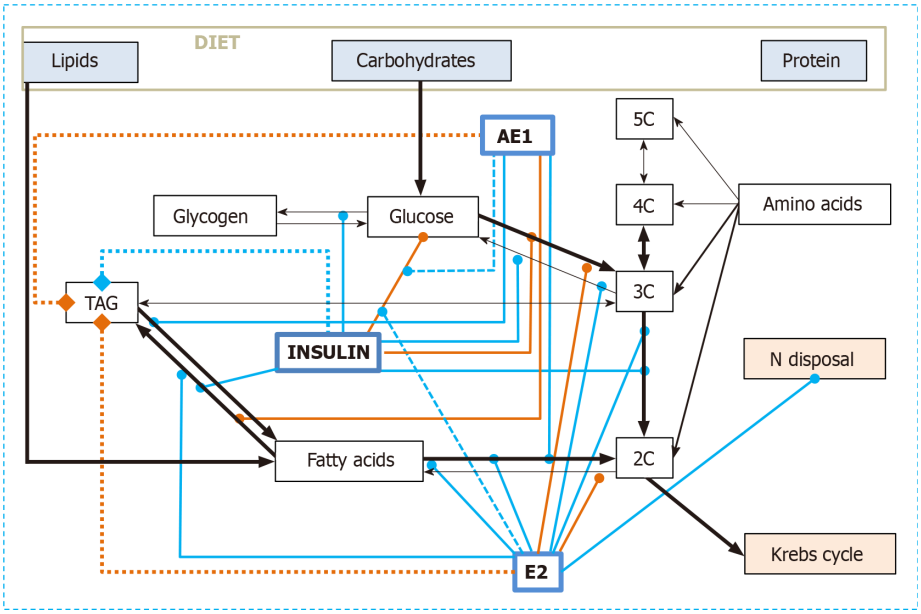

Figure 2 Combined regulatory effects on the utilization of dietary nutrients as energy substrates of insulin, 17β-estradiol and acyl-estrone. Black arrows: Metabolic relationships; solid green lines: activation/enhancement; solid red lines: deactivation/inhibition. Dashed green lines show activation effects on regulatory processes. Thicker dot lines with final diamond symbols represent the enhancement (green) or decrement (red) of TAG reserves. For the sake of simplicity, other regulatory agents are not shown, and neither are their interactions. AE1: Acyl-estrone; E2: 17β-estradiol and other E2-group estrogens

inflammation, excess fat accrual (and thus, obesity), and easing nitrogen excess normalization. This short (and incomplete) list is quite similar to a prescription for preventing the development of MS. Abundant epidemiological and limited experimental data support this assertion. It seems that we have to look more openly at estrogen forms to better understand their nature and properties, and to use them to fulfill their natural purpose as wardens of energy homeostasis (Box 5).

BOXES

Box 1 extended negative opinions on "sex hormones" hamper their investigative study and clinical development

In the case of "sex hormones" (*i.e.*, estrogens and androgens), the intensive pharmacological development has not displaced from use the most representative natural hormones: E2 and T from the front line of pharmacotherapy. However, the abuse of synthetic drugs (anabolic steroids, for instance) for purposes not strictly medical[352,353] have clouded the relatively recent recovery of T as a critical hormone for energy-partition[354,355].

The widely extended negative opinion against "sex hormones" continues to seriously hinder the use of T in the treatment of aging- and MS-related hypogonadism in mature and old men[225,356]. The "opinion war" against estrogen is, currently, even harder to overcome, because of its direct implication on women's sex; and because the social, political, and even religious arguments coalesced to raise questions (real or inflated[357]) against their use for any purpose outside a few restricted and socially-conditioned gynecologic disease applications[358-360]. The fact that the natural hormone E2 continues to be the main (and effective and cheap) estrogen standard drug only adds to the widely extended negative bias against estrogens[205,361].

Box 2 critical methodological questions - the differences between species

Most of the problems caused by pharmacological overdosing of androgenic anabolic drugs or estrogens are probably secondary to blocking the hypothalamus-hypophysis-gonadal axis, a possibility observed many years ago[362], but seldom taken into consideration in clinical practice (and even less when the use is not medically justified). Because of this problem, continuous administration of excess anabolic androgens, can result in the loss of reproductive function[363] in addition to the derangement of their regulative metabolic (or/and psychological) functions.

Most of the metabolic studies on estrogens have been carried out in rodents for obvious reasons, but there are clear differences in the estrogen (and androgen) functions in rodents from those in humans. This includes a number of aspects, starting with the most obvious: size, metabolic rate and lifespan. The duration of the reproductive cycle, "estrus" or "heat" of rats and mice is shorter than the human ovarian cycle (which incorporates parts of the estrus cycle[364]), but their extension, phasing and physiological structure are different. The estrus is observed in most mammalian species (not in humans and apes), and is marked by changes in body temperature and energy expenditure[365]. Size affects energy expenditure (allometry) [366] and lifespan[367]. The fact that women are usually uniparous and rats normally have a two-digit number of pups (requiring a much higher energy and nutrient supply effort at the expense of the dam) is also a quantitative difference that makes uncertain the direct comparison of hormone changes and their timing, and of substrate dynamics, between different species and reproductive cycle models. Another key difference, explained above, is the E2 (and T) transport in plasma. Humans carry E2 and T bound to SHBG in high proportions of total circulating hormone, but SHBG is absent in mature rodents.

Box 3 steroid hormones as medium-term signals focused to control gene expression

Steroid hormones have longer circulating-lives than most other hormones (or other signaling molecules), which are rapidly produced (or released), then act and are inactivated, all in a short time-span. The maintenance along time of rapid-response signals is kept thanks to repeated secretion-activation/inactivation-destruction processes, which allow for rapid regulation changes, again in a short period of time. Steroid hormones, however, are produced and secreted to last for much longer periods; their prime mechanism of action is, essentially, gene translation, a process which requires more intensity of signal and longer stimulation periods. The advantage of steroids is their unbeatable stability over time in comparison to short term-effect peptidic hormones, catecholamines, *etc.* The target tissue specific needs are adjusted through the expression of different receptors and signaling pathways for the same steroid hormone, with additional modulation of expression, or by the numbers or proportions of molecular species, allowing for further modification of their effects under changing conditions.

Box 4 estrogen structural resilience and the environment

Estrogenic signaling is very ancient, affecting quite a number of phyla as observed by the use of estrogen analogs in the context of co-evolution of plant allopathic defense against herbivores[10,368]. The estrin nucleus is highly resistant to environmental oxidation or bacterial catabolism[369]. The non-biological disposal of human waste induces the accumulation of estrogens in continental waters[370] and its sediments [371]; they are also found in sea sediments[372]. Persistence of estrogen in the natural medium has another negative aspect, the environmental effects of waste estrogen [373], affecting both invertebrates and vertebrates[8]. Human and domestic animal overpopulation extends the increase of estrogenic waste problem to become a serious health, ecologic and economic[8,374] problem that should be understood and adequately addressed.

Box 5 perspectives: the need for further advance in our knowledge of estrogens

A critical point for the continued use of estrogens in medicine is that of the extended use of molecules designed for specific clinical applications[375]. All these patented molecules do mimic some aspects of physiological estrogen actions, but not all of them [375]. The synthesis of estradiol analogs has been oriented to increase only some effects, sought for specific clinical applications[9,376]. However, most of these compounds were designed before our knowledge of estrogen function, mechanisms of action and metabolic effects were fully known[377]. In fact, right now, our knowledge of the full list of estrogen effects is incomplete. For instance, the role of estrogens in the control of brain organization[378,379], adjustment of the immune response[380,381], or even the estrogen function in core energy metabolism regulation[15].

The proof of our limited knowledge is the lack of a clear picture of all the physiological actions carried out by estrogens. Thus, how can we expect to extend this needed knowledge to drugs devised with specific (not global) objectives? How to test their effects on functions that so far have not even been uncovered or analyzed in the classical estrogens? In fact a similar caveat should be applied to all other steroid hormones, especially corticosteroids and androgens.

We are aware that the binding modulation of the effects of estrogenic drugs is unclear, especially as to which real estrogen (as a whole) actions are carried out by each of these compounds[382,383]. In any case, during the last 70-90 years, the use of synthetic estrogens has been slowly substituted by natural estrogens[383,384], with the practical abandonment of the wonder drug diethylstilbestrol. The case of anabolic androgens (and testosterone itself) is paradigmatic: the continued use of these drugs results at least in infertility[385] and cardiovascular damage[386]. The continued use of estrogens, in particular powerful analogs, may result in unexpected, possibly negative or unexplained effects[384,387,388], simply not detected because the protean nature of estrogenic action has not permeated yet to clinical (and, especially, pharmacological) practice.

We are uncovering the proverbial tip of the iceberg of a group of steroid hormones; we need a deep analysis of estrogen functions, both from the molecular and regulatory aspects, but never forgetting that steroids act on the whole body not only on specific organs and single isolated pathways. The actual role of hormone carriers in plasma (*i.e.* SHBG), the cyclic hypothalamic-hypophysis-gonadal axis function, and the possible disorders induced by estrogenic drug substitutes, must be studied and adapted to human physiology in order to be able to resolve endocrine disorders as a whole. First: do no harm; the effects of estrogens and analog drugs under clinical conditions must be known and fully evaluated because not all estrogens and related drugs act the same way[383,384].

Last, but not least, the real and realistic implication of estrogens (as well as androgens and corticosteroids) in the regulation of the metabolic hub of energy partition should be clarified. This is an essential step to limit the ravages of aging and to understand (and correct) disorders such as the widely extended MS.

CONCLUSION

The main conclusion of this review is, precisely, the paramount metabolic importance of the estrogens. This is based on the synergy between the different forms of estrogen to maintain energy (including, obviously, glucose homeostasis), whilst preventing oxidative damage, lipid-induced inflammation, excess fat accrual (and thus, obesity), and easing nitrogen excess normalization.

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REVIEW

Role of nucleic acid sensing in the pathogenesis of type 1 diabetes

Darshan Badal, Naresh Sachdeva, Deep Maheshwari, Preetam Basak

ORCID number: Darshan Badal 0000-0001-7582-6366; Naresh Sachdeva 0000-0001-6289-4749; Deep Maheshwari 0000-0003-4512-7948; Preetam Basak 0000-0003-3792-7929.

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Darshan Badal, Department of Pediatrics, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India

Naresh Sachdeva, Deep Maheshwari, Preetam Basak, Department of Endocrinology, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India

Corresponding author: Naresh Sachdeva, MSc, PhD, Additional Professor, Department of Endocrinology, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012, India. naresh_pgi@hotmail.com

Abstract

During infections, nucleic acids of pathogens are also engaged in recognition *via* several exogenous and cytosolic pattern recognition receptors, such as the toll-like receptors, retinoic acid inducible gene-I-like receptors, and nucleotide-binding and oligomerization domain-like receptors. The binding of the pathogen-derived nucleic acids to their corresponding sensors initiates certain downstream signaling cascades culminating in the release of type-I interferons (IFNs), especially IFN-α and other cytokines to induce proinflammatory responses towards invading pathogens leading to their clearance from the host. Although these sensors are hardwired to recognize pathogen associated molecular patterns, like viral and bacterial nucleic acids, under unusual physiological conditions, such as excessive cellular stress and increased apoptosis, endogenous self-nucleic acids like DNA, RNA, and mitochondrial DNA are also released. The presence of these self-nucleic acids in extranuclear compartments or extracellular spaces or their association with certain proteins sometimes leads to the failure of discriminating mechanisms of nucleic acid sensors leading to proinflammatory responses as seen in autoimmune disorders, like systemic lupus erythematosus, psoriasis and to some extent in type 1 diabetes (T1D). This review discusses the involvement of various nucleic acid sensors in autoimmunity and discusses how aberrant recognition of self-nucleic acids by their sensors activates the innate immune responses during the pathogenesis of T1D.

Key Words: Nucleic acid sensing; Type 1 diabetes; Pattern recognition receptors; Nucleic acid receptors; Type 1 interferon; Beta cells

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Core Tip: Under abnormal physiological conditions, such as excessive cellular stress or apoptosis, endogenous self-nucleic acids like DNA, RNA or mitochondrial DNA accumulate in extranuclear compartments or extracellular spaces or form complexes with host proteins. Such situations sometimes lead to the failure of discriminating mechanisms of nucleic acid sensors leading to proinflammatory responses as seen in autoimmune diseases like systemic lupus erythematosus, psoriasis and to some extent in type 1 diabetes (T1D). The understanding of the role of nucleic acid-sensing and their downstream signaling pathways is gradually evolving and provides another avenue in exploring therapeutic options for treating autoimmune diseases like T1D.

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INTRODUCTION

Type 1 diabetes (T1D) is a complex autoimmune disorder that involves infiltration of innate and adaptive immune cells culminating in the killing of insulin producing beta (β)-cells, mainly through T-cell dependent mechanisms. Pathogenesis of T1D involves an initial infiltration of mononuclear cells consisting of neutrophils, dendritic cells (DCs) and macrophages[1] in the pancreatic islets[2] followed by lymphocytic infiltration[3]. Beta-cell death is mainly mediated by autoreactive CD8+ T cells that release cytolytic granules, perforins facilitating the entry of granzymes in target β-cells[4,5]. The innate immune cells carry a variety of specialized receptors known as pattern-recognition receptors (PRRs) whose main function is to detect well-conserved structural motifs that are indispensable to pathogen survival and are known as pathogen-associated molecular patterns (PAMPs)[6]. In addition to recognizing PAMPs, these receptors under certain circumstances can also recognize damage associated molecular patterns (DAMPs) released by dying autologous cells, including β-cells, and can activate signaling cascade in a fashion similar to PAMPs recognition [7]. This recognition initiates a canonical immune signaling cascade driven by type 1 interferons (IFNs), mainly IFN-α to induce IFN-stimulated genes (ISGs) which activate inflammatory mediators, release cytokines responsible for instituting an inflammatory state in the pancreatic islets, and overexpression of HLA class-1 molecules on β-cells that enhances uptake of autoantigens by antigen-presenting cells (APCs)[8-10]. Nucleic acids, like other PAMPs, are vital for the survival and propagation of pathogens, and hence, the PRRs of the human innate immune system were evolved to recognize and mount an appropriate response against the pathogens bearing them. In various autoimmune conditions, like systemic lupus erythematosus (SLE), psoriasis, *etc.* and to some extent in T1D, the nucleic acids released by self-cells under certain physiological conditions, such as inflammation, stress, apoptosis, necrosis, pyroptosis, necroptosis, and NETosis act as ligands of PRRs, leading to either initiation of these autoimmune conditions or worsening of their pathogenesis[1,11,12]. In this review, we have summarized the recent advances in understanding the role of self-nucleic acids, their sensors, and downstream signaling pathways involved in the pathogenesis of T1D and discussed the novel therapeutic approaches targeting autoimmune diseases, including T1D.

NUCLEIC ACID SENSING

As a part of the innate immune system, PRRs are the primary sentinels against the microbes, and initiation of immune responses through PRR recognition is crucial for the host defenses. PAMPs, such as viral or bacterial nucleic acids, in addition to other bacterial or fungal cellular components, are commonly recognized by the host PRRs. Recognition of PAMPs by PRRs initiates a downstream signaling cascade resulting in the innate immune responses by promoting the expression of pro-inflammatory cytokines, IFNs, *etc.*[13]. These cytokines signal the adjacent cells to promote the

expression of various ISG to impair replication of pathogens. Besides microbial infection, PRRs activation by nucleic acids can also be initiated by the host cells. Stress or cell-death induced release of self-nucleic acids, such as genomic DNA, mRNA, tRNA and mitochondrial DNA (mtDNA) can also be recognized by PRRs to trigger inflammatory cytokines and type-I IFN, leading to chronic inflammation. Inappropriate or prolonged detection of these nucleic acids has been shown to be associated with many autoimmune diseases[11]. Presently, PRRs are classified into 4 main categories as follows: Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), absent-in-melanoma (AIM)-Like Receptors (ALRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), and C-type lectins (CTLs). CTLs and most TLRs are located in the plasma membrane, while the NLRs, RLRs, ALRs and a few TLRs are located intracellularly[13].

TLRs

TLRs are a conserved class of PRRs belonging to the family of type-I transmembrane receptor proteins consisting of an extracellular Leucine-Rich Repeat (LRR) domain and an intracellular C-terminal toll/IL-1 receptor (TIR) domain[14]. This domain is required for the interaction and recruitment of various adaptor molecules to activate downstream signaling pathways involving the transcription factors Activator Protein-1 (AP-1), Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB), and Interferon Regulatory Factor (IRF)[15]. To date, 13 different types of TLRs (TLR 1-13) have been identified. TLRs 1-9 are expressed by both humans and mice; whereas only humans, express TLR10, while mice are known to express TLR11-13[16]. TLRs are broadly expressed in both immune and non-immune cells in two distinct cellular compartments, extracellular and intracellular (mainly in endosomes)[17]. In T1D, upon recognition of pathogenic and/or foreign material, TLRs influence many immunologic mechanisms, including activation and maturation of APCs, antibody production, down regulating regulatory T cell (Treg) responses, and facilitating a pro-inflammatory environment through the secretion of a plethora of cytokines and chemokines [18].

TLR-TLR ligation and interaction transduces signals through MyD88 (Myeloid differentiation primary response 88)-dependent or independent pathways. Upon activation, MyD88 recruits Interleukin 1 Receptor Associated Kinase (IRAK-1), IRAK-4, and Tumor Necrosis Factor receptor (TNFR)-Associated Factor 6 (TRAF-6), which then activate c-Jun N-terminal Kinase (JNK), Ikβ Kinase (IKK), AP-1, and NF-κB. The MyD88-independent pathway is mediated by TIR-domain-containing adapter-inducing IFN-β (TRIF) and TRIF Related Adaptor Molecule (TRAM), leading to the activation of NF-κB, AP-1, or IRFs[19], while the TLR3 signaling is mediated through TRIF, TLR7, TLR8, and TLR9 signals through MyD88. It has also been demonstrated that TLR signaling can efficiently promote the uptake of autoantigens by APCs[8-10]. Under normal physiological conditions apoptotic cell derived antigens are not presented efficiently by MHC class II molecules. However, TLR ligand co-administration not only enhances antigen presentation but also promotes antigen specific responses by CD4+ T cells[8]. Thus, it means that TLRs not only acts as danger signal sensors but also regulators of self-and non-self-antigen discrimination[20,21]. In support of this fact, it has been demonstrated that stimulation of TLRs enhances antigen processing by up-regulating scavenger receptors *via* the MyD88-dependent pathway[22].

The role of TLRs especially those involved in the recognition of nucleic acids is also being recognized in autoimmune diabetes. TLRs can recognize various forms of endogenous DNA or RNA produced during virus infection induced cell death[23]. However, TLR3, TLR7, TLR8, and TLR9 specifically recognize viral-associated nucleic acids with comparatively higher affinity and have been implicated in the pathogenesis of T1D. TLR3$^{-/-}$ NOD mice have shown high mortality from Coxsackie B4 virus (CVB4) infections and the few that survived develop T1D[24]. Certain polymorphisms in the *TLR3* gene (rs3775291 and rs13126816) have also been shown to be related with a higher risk of T1D and a more aggressive pathology[25]. A double stranded RNA (dsRNA) mimetic polyinosinic: polycytidylic (poly I: C) has been reported to be recognized by TLR3, leading to induction and increase in the severity of T1D in mice, depending on dose and administration[25].

Stimulation of TLR7 (in addition to CD40 activation of DCs) can induce diabetogenic cytotoxic CD8+ T cells in the pancreatic lymph nodes of NOD mice to promote the onset of autoimmunity[26]. Repeated topical administration of a TLR7 agonist, imiquimod, is sufficient to promote T1D development while inhibition using IRS661 can significantly lower disease onset[26]. Similarly, TLR7 signaling in plasmacytoid DCs (pDCs) triggers B and T cell activation *via* IFN-I secretion in

rotavirus infections, on the other hand, inhibition of TLR7 can block this process and prevent the acceleration of T1D following infection[27]. Zhang *et al*[28] have shown that TLR9 blockade can impede the activation of diabetogenic CD8+ T cells and, delay autoimmune diabetes in NOD mice. Liu *et al*[29] generated TLR9 knockout NOD mice and observed improvements in insulin secretion, glucose tolerance, and β-cell function. These improvements were partially mediated by the upregulation of CD140a on β-cells. Similar results have been observed by the use of TLR9 antagonists or by genetic targeting on ontogenesis and function of β-cells to protect NOD mice from T1D.

Hence, these and other reports further necessitate more research to understand and improve defects associated with self-nucleic acid recognition by TLRs associated with T1D pathology.

RLRs

RLRs are a group of intracellular receptors that recognize viral dsRNA and are comprised of 3 proteins: (1) RIG-1; (2) Melanoma differentiation-associated gene 5 (MDA5); and (3) Laboratory of genetics and physiology 2 (LGP2), which is composed of a DExD/H box RNA helicase domain and a C-terminal domain[30]. Both RIG-1 and MDA5 contain additional N-terminal caspase activation and recruitment domains (CARDs) that transmit downstream signaling. RIG-I and MDA5 have similar functions and they initiate antiviral signals to induce *IFN* gene activation, while LGP2 acts as a regulator of MDA5 and RIG-1[31]. Upon recognition of RNA, an ATP-dependent conformational change occurs in RLR[32] resulting in the activation of CARD and further activation of an adaptor molecule, mitochondrial antiviral signaling (MAVS) protein[33]. Activation of MAVS, in turn, triggers signaling cascades involving TRAF3/6, caspase 8/10, RIP-1, fas-associated death domain, and TNF receptor-associated death domain ultimately activating TANK binding kinase 1 (TBK1)/IKK-ε and IKKα/IKKβ to induce transcription of type-I IFNs and proinflammatory cytokines by activating IRF-3 and NF-κB.

When challenged with pathogenic stress, various single nucleotide polymorphism (SNP) in the *interferon induced with helicase C domain 1* (*IFIH1*) gene have been found to cause greater or reduced susceptibility in the pathogenesis of T1D *via* altering MDA5 activation and expression[34]. The *IFIH1* mutation A946T (rs1990760) has been involved in the pathogenesis and development of various autoimmune diseases like T1D, SLE, and multiple sclerosis (MS)[35,36]. Two independent studies conducted on subjects with diabetes showed that subjects with heterozygous A946T SNP have a more prominent immune response and ISG expression to Coxsackie virus challenge in comparison to healthy controls, suggesting greater IFNs and ISGs expression during infection[37,38]. In another study, Cinek *et al*[39] demonstrated a positive correlation between IFIH1 polymorphism (rs1990760), which is known to be strongly associated with T1D, and enteroviral RNA frequency in the blood of T1D subjects. The authors further suggested that rs1990760 can modify enteroviral frequency in the blood of healthy children harboring IFIH1 polymorphism, predisposing them towards T1D [39]. Gain-of-function mutations in *IFIH1* have been also found to be associated with overexpression of type 1 and type 3 IFN[40]. A study by Gorman *et al*[41] observed mice that were homozygous for *IFIH1* SNP (946T) or exhibiting IFIH1 risk alleles (843R and 946T) simultaneously, had enhanced expression of *IFIH1*-related genes, increased rate of autoimmunity development, and ability to recognize self-RNA. Such mutations may alter the expression of inflammatory molecules and the dynamics of target binding, and activation may also be altered, resulting in more potent/enhanced IFN response leading to the risk of T1D. For example, MDA5 mutation E627 causes loss of a portion of C-terminal region, resulting in loss of dsRNA ligand and binding[42]. Overall, these reports provide us with enough knowledge about the role of RLRs in the pathogenesis of T1D.

ALRs

A few PRRs also include some members of the family of proteins containing pyrin and hematopoietic interferon-inducible nuclear (HIN) domain[43]. The Pyrin and HIN domain (PHYIN) family of proteins comprises of ALR, which contains an N-terminal Pyrin domain and one or two C-terminal hematopoietic IFN-inducible nuclear proteins with 200 amino acids (HIN-200) domains, containing an oligonucleotide/oligosaccharide-Binding fold (OB fold), which is a common DNA-binding motif [44]. Of all ALRs, absent in melanoma 2 (AIM2) protein is the only one conserved in both humans and mice. AIM2 possesses the ability to sense DNA in the cytoplasm and as well as in the nucleus[44].

AIM2 is a cytosolic dsDNA receptor that oligomerizes on recognizing cytosolic foreign dsDNA and promotes the polymerization of the adaptor protein, Apoptosis-associated Speck-like (ASC) protein and eventually forming a caspase-1 activating inflammasome[44]. AIM2 binds to small DNA fragments up to 20bp; however, in order to initiate immune responses against longer DNA fragments, oligomerization of AIM2 is required. ALRs can sense self-DNA through leakage from nuclear envelope and exosomes engulfed by phagocytes; however, the ability of ALRs to elicit type 1 IFN responses is questionable, as mice deficient in ALRs can mount effective type 1 IFN responses to DNA viruses and lentiviruses[45].

NLRs

NLRs are comprised of various cytosolic PRRs, which are characterized by the presence of a conserved NOD[46]. NLRs consist of an N-terminal effector binding region, which is further comprised of: (1) Protein-protein interaction domain such as the: (a) CARD; (b) Pyrin domain (PYD); and (c) Baculovirus inhibitor repeat domain; (2) NOD domain, which is needed for self-oligomerization and nucleotide binding; and (3) Array of C-terminal LRR motifs to recognize the pathogenic pattern and regulate NLR activity.

Upon recognition of nucleic acids by the C-terminal LRR motifs, the downstream signaling gets initiated, involving conformational changes that result in oligomerization of NLR *via* the NOD domain. NLR exposes the effector domains to initiate CARD and PYD recruitment and activation by enhancing their oligomerization[47]. NLRs interact with receptor interacting serine/threonine protein kinase 2 to trigger mitogen-activated protein kinase (MAPK) and NF-κB[48]. The NLRs have a proven role in antiviral immunity; however, their role in sensing self-nucleic acids is gradually emerging[49]. NLRs also recognize oxidized forms of mitochondrial DNA, which could have important implications in inflammation and cancers[50].

Role of ALRs and NLRs in the formation of inflammasomes

Inflammasomes are a diverse class of cytosolic multiprotein complexes consisting of an adaptor protein containing CARD, a sensor protein and caspase-1 which is highly proinflammatory. Their assembly can be triggered by a variety of stimuli, ultimately leading to caspase-1 activation and synthesis of proinflammatory cytokines. Inflammasomes play a crucial role in the mobilization and activation of various immune cells in maintaining tissue homeostasis by initiating acute immune responses. Inflammasomes can also initiate chronic immune response leading to uncontrolled inflammation which eventually causes cell death *via* pyroptosis[51]. Among them, NLRP3 and NLRP1 inflammasomes are the most common subtypes[52]. ALRs and NLRs initiate the immune response by forming inflammasomes, thereby alleviating IL-1β and IL-18 maturation and release[53,54]. Activated caspase-1 then cleaves pro-IL-1β or pro-IL-18 ,enabling the release of the mature active cytokines IL-1β and IL-18[53,55].

NLRP3 inflammasomes have been reported to play crucial roles in the pathogenesis of various autoimmune disorders, including T1D[56,57]. In 2019, Sun *et al*[58], showed the association of SNPs with T1D pathogenesis and diabetes onset in the *NLRP1* gene of T1D patients of Chinese Han origin. Increased susceptibility to T1D and celiac disease have been reported to be associated with SNPs within the *NLRP3* gene. A study by Hu *et al*[59] showed an important role of NLRP3 in the pathogenesis of T1D in NOD mice. Elimination of NLRP3 altered T cell maturation *via* regulation of CCR5 and CXCR3 expression, as well as pathogenic T cell mobilization to the pancreatic islets, which is a crucial process leading to β-cell death and disease progression. Also, knockout of NLRP3 downregulated C-C motif chemokine ligand 5 (CCL-5) and C-X-C motif chemokine ligand 10 (CXCL10) expression in the pancreatic islets *via* IRF-1 signaling[59]. Furthermore, in STZ induced diabetic mice model, NLRP3 activation *via* mtDNA initiated IL-1β production in caspase-1 dependent manner, suggesting a direct role of NLRP3-caspase1 signaling in T1D[60]. Pereira *et al*[61] recently highlighted the role of mtDNA in the involvement of vascular endothelial dysfunction in human subjects with T1D and asserted on the connection between NLRP3 inflammasomes and T1D complications. In this study, mtDNA isolated from diabetic mice promoted NLRP3 inflammasome activation *via* mechanisms involving mitochondrial ROS and Ca^{2+} influx, which was abrogated in NLRP3 knockout mice.

Cyclic GMP-AMP synthase-stimulator of IFN

The cyclic GMP-AMP synthase-stimulator of IFN genes (cGAS-STING) is a DNA sensing receptor present in the cytoplasm that recognizes host/pathogenic DNA[62]. When DNA binds on the active site of cGAS, its C-terminal containing the catalytic

unit undergoes a variety of conformational changes, resulting in cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) formation from ATP and GTP [63,64]. cGAMP formation results in STING activation by inducing conformational changes upon binding to its active site and also facilitates STING transportation from the endoplasmic reticulum to the Golgi apparatus[65,66]. Upon activation, STING further forms a complex with TBK1, which further phosphorylates IRF3 in endolysosomes[67,68]. Phosphorylated IRF3 translocates into the nucleus undergoing dimerization, and thus inducing the expression of ISG[69,70]. However, STING is also involved in the stimulation of IFN-β by interacting with the Translocon-Associated Protein (TRAP)[65,71].

The role of cGAS-STING in various autoimmune disorders is being widely explored, while its role in T1D has not been reported earlier. Lemos *et al*[72] reported that the activation of STING resulted in suppression of T1D onset and progression when NOD mice were administered with DNA nanoparticles, which promoted indoleamine 2,3 dioxygenase (IDO) activity, thus modulating T cell immunity in pancreatic lymph nodes and pancreas.

Overall, many studies have yielded important information on how the nucleic acid sensors lead to the activation of downstream signaling pathways (Figure 1). These sensors and their signaling mediators have been implicated in different autoimmune diseases including T1D (Table 1)[73-84].

TYPE 1 IFN SIGNALING: AN IMPORTANT CONVERGING POINT

Most of the nucleic acid recognition pathways culminate in the release of type 1 IFN, especially IFN-α, *via* mediators like IRFs, which makes them one of the most crucial part of the nucleic acid sensing pathway. IFN-α has multiple roles, including upregulation of human leukocyte antigen (HLA) class I and HLA class II to enhance antigenic presentation, increase in immunoproteasome activity, induce ER stress and cellular inflammation through TYK2 activation, induction of transcription factors, and signal transducer and activator of transcription 2 and IRF9. It also acts synergistically with IL-1β and induces β-cell apoptosis[85]. Heightened IFN-α secretion in peripheral blood mononuclear cells of T1D subjects by stimulation with influenza viruses has been attributed to the recognition of viral nucleic acids by endosomal TLRs of pDCs. Additionally, *in vitro* studies have demonstrated that pDCs secreted IFN-α enhances Th1 responses[86]. Another study observed higher levels of secreted IFN-α by pDCs obtained from the relatives of T1D subjects following their stimulation with CpG 2216 [87]. The transition of prediabetic stage to full-blown diabetes is also found to be controlled by IFN-α signaling. The study demonstrated that the infiltration of autoreactive T cells and β-cell killing can be prevented by blocking IFN-α signaling by sphingosine-1 receptor agonist prior to the clinical onset of disease[88]. Rodrigues *et al* [89] in a recent study revealed IFN-1 hyper-responsiveness in T1D after innate immune stimulation of whole blood cells with CpG DNA. They observed higher induced IFN-1-associated gene expression in monocytes from NOD mice. Similarly, in human participants, *ex vivo* whole blood stimulation showed higher induced IFN-1 responses in participants with T1D compared with healthy controls. In our recent study, we, too, observed increased secretion of IFN-α by the peripheral pDCs from T1D subjects compared to non-diabetic controls. Enhanced IFN-α secretion was also observed after stimulation with DNA-LL37 complexes indicating the inflammatory nature of pDCs derived from T1D subjects. Collectively, these data support the notion that IFN-α mediated effects play an important role in the early pathogenic events during initiation of autoimmune diabetes, and the presence of early type 1 IFN signature in susceptible individuals and animal models suggests the role of viral nucleic acids, and to some extent, the self-nucleic acids in T1D pathogenesis.

SELF-NUCLEIC ACIDS: ROLE IN PATHOGENESIS OF TYPE 1 DIABETES

During the initial phase of T1D, innate immune cells, like DCs, neutrophils and macrophages, infiltrate the islets much before the infiltration of T and B cells[2,90-92]. This buildup of innate immune cells is persistent during the later β-cell destructive insulitis as well[93]. Therefore, the entry of DCs and macrophages/monocytes can be considered an initial sign of the autoimmune process during the pathogenesis of T1D [1,20,94].

Table 1 Nucleic acid sensors involved in various autoimmune diseases including type 1 diabetes

No.	Nucleic acid sensor	Downstream signaling molecule	Autoimmune disease	Ref.
1	TLR9	Myd88/ IRF3/7	SLE	[73,74]
2	TLR7	Myd88	T1D	[27]
3	TLR3	TRIF	T1D	[25,75]
4	RLR	IRF3	Singleton-Merton Syndrome,	[76,77]
			AGS and T1D	[34,78]
5	cGAS-STING	cGMP	SLE and AGS	[79,80]
6	NLR	Inflammasome activation	T1D and SLE	[58,81]
7	AIM		SLE	[81]
8	IFI16	Inflammasome	Primary Sjogren's Syndrome	[82]
		Activation	Rheumatoid Arthritis	[83]
9	CTL	Bcl10/CARD9	Multiple Sclerosis	[84]

AIM: Absent-in-melanoma; cGAS-STING: Cyclic GMP-AMP synthase-stimulator of interferon genes; CTL: C-type lectin; NLR: Nucleotide-binding and oligomerization domain-like receptor; RLR: Retinoic acid inducible gene-I-like receptors; TLR: Toll-like receptor; TRIF: TIR-domain-containing adapter-inducing IFN-β.

Although there is ambiguity regarding the exact role of innate immune cells and other initial triggers involved in the loss of β-cell tolerance, certain factors, like viral infection and ER stress are known to provoke an immune response in β-cells leading to the activation of pro-inflammatory pathways. Additionally, β-cells themselves might also participate in their demise by invoking apoptosis rather than being an innocent victim of autoimmune attack as previously thought [95]. One of the outcomes of β-cell destruction is the release of self-nucleic acids along with other cellular debris. Among the nucleic acids, the role of self-DNA in the development of T1D is highlighted by few studies, Diana *et al*[1] demonstrated that neutrophils, B-1a cells, and plasmacytoid dendritic cells are recruited to islets during physiological periods of β-cell death. Activated B-1a cells secrete dsDNA specific IgGs, which activate neutrophils to release DNA-binding cathelicidin-related antimicrobial peptide (CRAMP), which binds self-DNA, and along with DNA-specific IgG, activating pDCs through the TLR9–MyD88 pathway, leading to IFN-α production in pancreatic islets and initiation autoimmune diabetes in NOD mice. Mollah *et al*[96] observed increased incidence of diabetes associated with increased accumulation of ssDNA in the immune cells of granzyme A (protease degrading intracellular DNA) deficient NOD mouse due to induction of IFN response in pancreatic islets. The study identified DNA as a novel endogenous trigger of autoimmune diabetes and an *in vivo* role for granzyme A in maintaining immune tolerance. Earlier, Zentsova *et al*[97] had also observed that monocytes contribute to DNA sensing in patients with T1D *via* the TBK1 and STING pathways by recognizing CpG-DNA leading to the release of IFN-α and proinflammatory cytokines. These studies highlight the importance of investigating the interaction of DNA sensors of innate immune cells during the early pathogenesis of T1D. However, limitations in obtaining pancreatic tissues pose a big challenge in assessing such interactions.

Besides DNA, the role of self-RNA in the progression of T1D is also being speculated. A study by Kocic *et al*[98] demonstrated that accumulation of circulating self-RNA can lead to the progression of autoimmune or inflammatory conditions in subjects with juvenile T1D. Recently, studies from several groups suggested that adenosine deaminase acting on RNA (Adar1) deficiency leads to the accumulation of retroelements, such as Alu:Alu hybrids, in the cytoplasm, which are then recognized by MDA5, resulting in excessive proinflammatory response[99,100]. Furthermore, mouse models deficient in Adar1 established that dysregulated RNA editing caused MDA5-driven autoimmunity[101,102]. The role of mtDNA acting as a ligand for nucleic acid sensors is also being observed by various research groups. When mtDNA is released into extracellular space and cytoplasm, it activates a variety of innate immune responses. West *et al*[103] showed that the mitochondrial transcription factor A (TFAM) deficiency leads to mis-packaged mtDNA, resulting into its cytoplasmic release where it bound and activated cGAS initiating a type-I IFN response. mtDNA has also been involved in the activation of inflammasome[104]. Carlos *et al*[105] shown

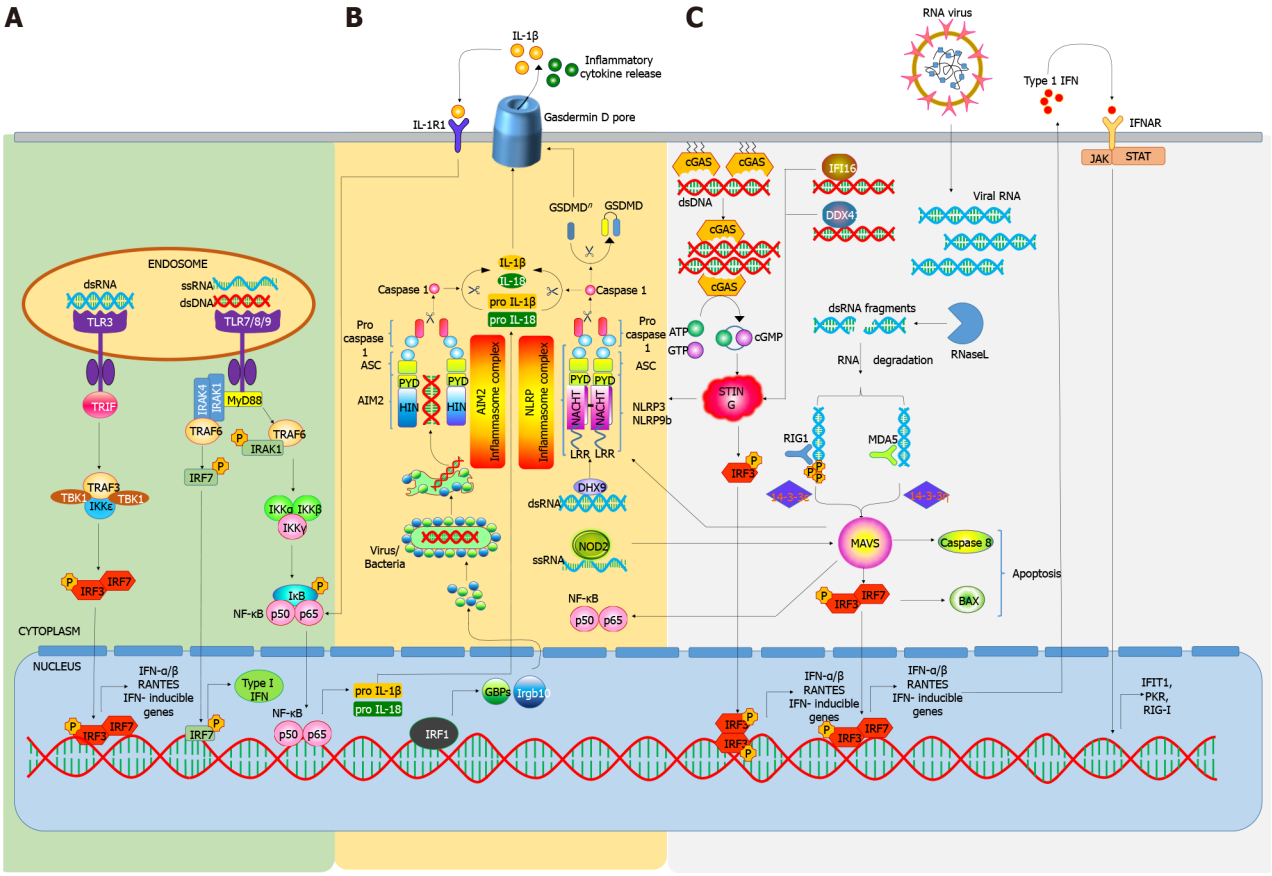

Figure 1 Nucleic acid sensors and their signaling pathways involved in autoimmune diseases including type 1 diabetes. A: Toll-like receptor (TLR) signaling: Priming of nucleic acid sensing is mediated by the activation of several TLRs, which are located in endosomes. For *e.g.*, TLR3 recognizes double stranded RNA initiating downstream TIR-domain-containing adapter-inducing interferon (IFN)-β dependent signaling cascade *via* activation of IRF3 and IRF7, resulting in the induction of IFN-stimulated genes (ISGs). On the other hand, TLR7, TLR8 and TLR9 recognize ssRNA and dsDNA to trigger downstream signaling *via* Myd88, resulting in higher expression of either type1 IFNs or NF-κB *via* IRF7 and IκB phosphorylation, respectively. NF-κB activation further stimulates the production of pro interleukin (IL)-1β and pro IL-18, which get cleaved by caspase 1 into mature IL-1β and IL-18, respectively; B: Inflammasome complexes: Following recognition of nucleic acids, recruitment of various adaptor proteins occurs to form mature inflammasome complexes, which further cleave pro-caspase 1 and gasdermin D (GSDMD) into active caspase 1 and GSDMD^{n} (GSDMD n-terminal), respectively. GSDMD gets inserted into the plasma membrane and helps in the release of inflammatory cytokines; C: Cytosolic Receptors: cGAS is another DNA sensor localized close to the plasma membrane. It recognizes and forms complexes with dsDNA. cGAS-dsDNA binding induces the catalytic synthesis of cGAMP from ATP and GTP, which further culminates in the stimulation of STING. Other DNA binding proteins (or sensors) like IFI16 and DDX41 also recognize DNA and activate STING, which further facilitates NLRP inflammasome activation. STING also activates the battery of IFN genes *via* IRF phosphorylation. Different forms of RNA originating from wide sources, like viral RNA, degraded self-RNA, *etc.* are recognized by RLRs, including RIG-1 and MDA5, following which they are imported to mitochondrial antiviral signaling (MAVS). MAVS further activates ISGs *via* IRF3-IRF7 activation. IFNs also work in an autocrine fashion and stimulate more production of different nucleic acid sensors and other ISGs. AIM2: Absent in melanoma; ASC: Apoptosis-associated speck-like protein containing a CARD (Caspase activation and recruitment domain*)* Domain; BAX: Bcl-2-associated X protein; cGAS: Cyclic GMP-AMP synthase; DDX41: DEAD-Box helicase 41; DHX: DEXH-box helicase; GBP: Guanylate-binding proteins; GSDMD: Gasdermin D; GSDMD^{n}: Gasdermin D (N-Terminal); HIN: Hematopoietic IFN-inducible nuclear protein; IFI16: Interferon gamma inducible 16; IFIT1: Interferon induced *protein* with tetratricopeptide repeats 1; IFN: Interferon; IFNR: IFN receptor; IGRB10: Immunity-related GTPase family member B10; IKK: Ikb (Inhibitor of Nuclear Factor Kappa B) Kinase; IL: Interleukin; IL-1R1: IL-1 receptor 1; IRAK: Interleukin-1 receptor associated kinase; IRF: Interferon-regulatory factors; ISG: Interferon stimulated genes; JAK: Janus kinase; MAVS: Mitochondrial antiviral-signaling protein; MDA5: Melanoma differentiation-associated protein 5; Myd88: Myeloid differentiation primary response 88; NLRP: NLR (NOD-like receptor) family pyrin domain; NOD: Nucleotide binding and oligomerization domain; PKR: Protein kinase R; PYD: PYRIN Domain; RIG1: Retinoic acid-inducible gene I; STAT: Signal transducer and activator of transcription; STING: Stimulator of interferon genes; TBK1: TANK (TRAF family member-associated NF-kappa-B activator)-binding kinase 1, TLR: Toll-like receptor; TRAF: TNF (Tumor necrosis factor) receptor associated factors; TRIF: TIR [toll/interleukin-1 (IL-1) receptor] domain containing adapter inducing interferon-β.

that mtDNA activates NLRP3 to trigger IL-1β secretion *via* caspase-1-dependent pathway to precipitate the onset of streptozotocin (STZ) induced T1D in C57BL/6 mice. In 2020, Pereira *et al*[61] observed that mtDNA promoted NLRP3 inflammasome activation that contributed to inflammation and endothelial dysfunction in patients with T1D.

NUCLEIC ACID SENSING: WHAT LEADS TO THE DYSREGULATION?

The role of nucleic acids and their signaling has been explored by several studies in many autoimmune diseases, yet there is very little data on the aberrations in nucleic acid sensing mechanisms in autoimmune *vs* non-autoimmune conditions. Parallels are drawn from those autoimmune diseases, like psoriasis and SLE, where nucleic acids are targeted by the immune cells. During the pathogenesis of SLE, the pDCs get activated due to facilitated recognition of autoantibodies against nucleic acids by TLR7 and TLR9 leading to increased secretion of type 1 IFNs[106,107]. A similar role of self-DNA complexes and specific antibodies was also suggested by Diana *et al*[1] during the initial stages of T1D in the activation of TLR9 in pancreatic pDCs, which release IFN-α in NOD mice, as explained earlier.

An important study by Revelo *et al*[108], explored the possible role of different types of nucleic acids contributing to glucose intolerance during diet induced obesity (DIO). The study concluded that oxidized mtDNA derived from abnormal formation of extracellular traps (ETs) can promote inflammation of metabolic tissues *via* TLR7 and TLR9 in pDCs. The same study has also explored the possible role of exogenous sources of nucleic acids like CpG-ODN, which worsened glucose tolerance in lean mice, possibly by the recognition of CpG DNA by TLR9. A similar study has also shown that increased levels of circulating cell free DNA are involved in the activation of macrophages *via* TLR9 during DIO[109]. A recent study by Zentsova *et al*[97] demonstrated altered DNA sensing in subjects with T1D in response to microbial DNA. Prominent proinflammatory responses were observed in pDCs and monocytes of T1D patients compared to healthy controls. Furthermore, monocytes isolated from T1D subjects were shown to bind and internalize DNA and responded by releasing higher levels of proinflammatory cytokines as compared to control subjects. Surprisingly, this cytokine production was independent of the TLR9 signaling pathway but dependent on other intracellular receptors like, TBK1 and STING for recognition of CpG-DNA and NETs, which were used to mimic self-DNA in the study. During our study on the role of self-DNA in T1D, we have also observed that the pDCs and monocytes of T1D subjects behave differently from those of healthy subjects. We observed that the pDCs and monocytes of T1D subjects were more prompt on acquiring an inflammatory phenotype upon stimulation with molecules like DNA-LL37 complexes by initiating inflammation through IFN-α and augmenting autoimmunity by activating CD4+ T cells[110]. Therefore, it appears that either altered forms of nucleic acids or alterations in their sensors underlie the dysregulations in nucleic acid sensing in autoimmunity.

Formation of nucleic acid-protein complexes

In normal circumstances, the self-nucleic acids are considered non-immunogenic in nature and in the extracellular environment, they undergo rapid degradation by various extracellular nucleases[111]. However, their binding to peptides like, LL37 and HMGB1 (released by neutrophils and monocytes, respectively)[112,113] can lead to the formation of complexes that are resistant to nuclease degradation. These complexes are transported to endosomal compartments of pDCs and monocytes, which are recognized by TLR9[114]. In the case of NOD mice, CRAMP (mouse equivalent of LL37) is known to form complexes with self-DNA and DNA-specific IgG to induce IFN-α production *via* the TLR9 and MyD88 pathways. In T1D, we have also observed that LL37 forms stable complexes with self-DNA to protect it from DNase degradation and, at the same time, it increases the efficiency by which pDCs and monocytes engulf DNA complexes in their cytosol[110]. Moreover, delayed clearance of apoptotic cells and other cellular debris by the macrophages also causes their accumulation, which in turn results in increased uptake of nucleic acids by innate immune cells, like pDCs and DCs that express abundant nucleic acid sensors. Apart from self-DNA, self-RNA is also capable of forming stable immune complexes with LL37, which was first observed by some researchers where they observed stable formation of complexes that readily enter endosomes of both pDCs and mDCs to induce TLR7 activation that finally triggers IFN-α secretion. Taking cue from these aforementioned studies it can be concluded that self-nucleic acids, like RNA, DNA and mtDNA, that are released from the dying β cells can form complexes with certain peptides and activate innate immune cells like pDCs, DCs and macrophages, and tilting the local immune homeostasis towards proinflammation.

However, the main unanswered question that remains is how does the uptake of self-nucleic acids or their complexes with proteins confer a proinflammatory phenotype to innate immune cells like the uptake of nucleic acids of viral and bacterial origin. Comparative studies done in past have shed some light and indicated that self-

nucleic acids can induce similar if not heightened immune responses during the progression of autoimmune diseases, including T1D, although this hypothesis is still in its nascent stages and require some solid comparative studies, especially in T1D pathogenesis. The role of molecular mimicry by self-nucleic acids cannot be denied as they share similar motifs to pathogenic genomes like that of viruses and bacteria, a very good example of which is the presence of CpG islands in mtDNA. The role of nucleic acid induced innate immune inflammation also becomes particularly important, especially when viral infections alone cannot explain the initial infiltration and activation of innate immune cells, like pDCs, DCs, and monocytes, during the initial stages of T1D.

TARGETING NUCLEIC ACID SENSING PATHWAYS: THERAPEUTIC STRATEGIES

With the increasing understanding of their roles and the signaling cascades in initiating inflammatory responses, novel therapies involving PRRs, have been attempted to target autoimmune diseases (Table 2).

Historically, targeting of downstream TLR signaling pathways using antimalarial drugs like chloroquine, quinacrine, and hydroxyl-chloroquine (HCQ) have been used in the treatment of autoimmune diseases since the 1940s, suggesting the effectiveness and importance of blocking endosomal TLR signaling rather than blocking TLR ligand themselves[115]. Compared to HCQ, CpG-52364, a quinacrine derivative and small-molecule antagonist of TLR7/8/9 is therapeutically more effective and has fewer side effects in animal studies. A phase I clinical trial for treatment of SLE (NCT00547014) showed inhibition of disease development without causing general immunosuppression[116]. Next, the idea of reducing exogenous DNA and RNA associated DAMPs has also been tried as an alternative and broader approach to suppress non-TLR dependent pathways of IFN production for the treatment of autoimmune diseases. Pulmozyme, a recombinant human DNase, has been in use since 1994 for the treatment of cystic fibrosis[117]. Additionally, Macanovic *et al*[118] showed that murine DNase can improve renal histology in NZB/NZW F1 Lupus-prone mice. A bovine DNase preparation also had initial success in improving clinical outcomes in a patient trial of SLE, but further studies were precluded due to the development of antibodies to the bovine DNase[119].

Oligodeoxynucleotides (ODNs) were first designed for direct binding and for antagonizing endosomal TLRs as an alternate strategy to treat SLE, which despite showing initial success the therapy, failed to garner support due to several reports of adverse effects like thrombocytopenia and neutropenia. Although greater promise was shown by ODNs, like immunomodulatory oligonucleotides (IMO)-8400 in psoriasis that target TLR7, TLR8, and TLR9 to reduce the expression of IL-17 signaling associated genes[120,121]. A phase 2a clinical trial, sponsored by Idera Pharmaceuticals, involving use of IMO-8400 for the treatment of plaque psoriasis exhibited reduced psoriasis severity with good tolerance in the recruited subjects (NCT01899729)[122]. A preclinical study on INH-ODN-24888, a guanine modified oligonucleotide was initiated for the treatment of lupus patients based on its activity as a TLR7 and TLR9 antagonist, and it was observed to be more efficient than the unmodified oligonucleotide (INH-ODN-2088)[123,124].

Other peptide compounds designed to inhibit TLR signaling pathways in autoimmune diseases include SM934 (b-aminoarteether maleate). It targets TLR7 and TLR9 signaling cascades, thereby promoting their downregulation along with regulation of MyD88 expression and NF-kB activation through an unknown mechanism. Finally, it inhibits TLR-induced activation of B cells leading to a decrease in proliferation and antibody secretion in MRL/Lpr mice (animal model of SLE)[125]. Another peptide ST-2825 that blocks the dimerization of MyD88[126] by interfering with the recruitment of IRAK1 and IRAK4 to TLR7- and TLR9-MyD88 complexes was found to be of therapeutic importance in inhibiting TLR-mediated inflammatory responses. Recently, PF-06650833, a small molecule inhibitor of IRAK4 has been reported to be effective in ameliorating some symptoms in patients with moderate to severe rheumatoid disease[127]. Another molecule, reported as "Compound II" in the study by Hasan *et al*[128], was shown to inhibit TBK1 and consequently douse the hyper-inflammatory responses in Trex$^{-/-}$ mice. Another novel inhibitor, TJ-M2010-6, has also shown the ability to suppress homo-dimerization of MyD88 by interacting with amino acid residues of its TIR domain, thereby preventing and treating T1D in NOD mice. Upon deducing the mechanistic pathways, it was observed that TJ-M2010-

Table 2 Studies and trials with antagonists/inhibitors of nucleic acid sensors and their signaling mediators in various autoimmune diseases

No.	Inhibitor	Disease	Target	Phase (Preclinical/Clinical-trial ID)	Ref.
1	Hydroxychloroquine	Rheumatoid arthritis and SLE	TLR7, TLR9, cGAS-STING	NCT0380218 (Ongoing Trial)	[139]
2	SM934	SLE	TLR7 and TLR9	NCT03951259 (Phase II)	[125]
3	Amlexanox	T2D	TBK1 and IKKε	NCT01975935 (Phase II)	[140]
4	TJ-M2010-6	T1D	Myd88	Preclinical	[129]
5	ST-2825	SLE	IRAK1 and IRAK4	Preclinical	[126]
6	Aspirin	AGS	cGAS	Preclinical	[141]
7	ODN-1411	Rheumatoid Arthritis	TLR8	Preclinical	[142]
8	INH-ODNs	SLE	TLR3 and TLR9	Preclinical	[143]
9	X6	Autoimmune myocarditis	cGAS	Preclinical	[144]
10	PF-06650833	Rheumatoid Arthritis	IRAK4	NCT02996500 (Phase II)	[127]
11	Compound II	SLE and AGS	TBK1	Preclinical	[128]
12	Sifalimumab (MEDI-545)	SLE	IFN-α	NCT00979654 (Phase II)	[145]
13	AGS-009	SLE and Rheumatoid Arthritis	IFN-α	NCT00960362 (Phase I)	[132]
14	IMO-8400	Plaque Psoriasis	TLR-7, 8, and 9	NCT01899729 (Phase IIa)	[122]
15	CpG-52364	SLE	TLR-7, 8, and 9	NCT00547014 (Phase I)	[116]

SLE: Systemic lupus erythematosus; T2D: Type 2 diabetes; TID: Type 1 diabetes.

6 treatment prevents insulitis *in vivo*, whereas *in vitro* experiments showed inhibition of DCs maturation, leading to suppression of T cell activation and production of inflammatory cytokines[129]. To directly target the interaction of TLRs with their corresponding ligands, several antibodies have been designed, including Sifalimumab (NCT00979654, NCT01283139) and AGS-009 (NCT00960362). Both of the antibodies showed significant reduction of the IFN-α signature in the clinical trials aimed at SLE treatment[130-132]. However, despite the indispensable role of endosomal TLRs in the pathology of several type 1 IFN-driven autoimmune diseases, the therapeutic strategies against TLR7, TLR8, and TLR9 have yet to see appreciable success in various clinical trials.

Recent data on the involvement of molecular pathways leading to NETosis, and the components of NETs, like myeloperoxidase MPO, neutrophil elastase NE, and nucleic acids, have made them an attractive target for therapeutic strategies in autoimmune diseases, including T1D[133]. The best studied and the viable target is PAD4, which is a nuclear enzyme mediating NET formation by chromatin de-condensation[134], several inhibitors against NETs have been tried, of which GSK484 has shown persistent activity in animal models of inflammatory disease[135]. Additionally, an enzyme, staphylococcal nuclease, has shown some promise by degrading intestinal NETs and ameliorating both intestine and pancreatic islet inflammation to effectively regulate the blood glucose homeostasis in NOD mice[136]. Keeping in view the important roles played by nucleic acid sensing in shaping immune responses, specifically *via* modulation of innate immunity, researchers are actively exploring the nucleic acid-based nanoparticles that can be designed and functionalized with known therapeutic immunomodulatory domains and motifs, for the treatment of various nucleic acid centered autoimmune diseases[137,138]. Collectively, these studies emphasize the scope of further exploration of novel approaches to targeting key checkpoints in nucleic acid recognition and their downstream signaling pathways.

CONCLUSION

There are ample studies on T1D pathogenesis in both humans and animal models, and significant progress has been made in understanding the role of various cellular mechanisms involved in the initiation of the disease. Emerging data on the contri-

bution of nucleic acids and their receptors on innate immune cells is challenging the current dogmatic and historical view of T1D as being a T cell driven disease.

The evolving view, that we have tried to support in this review, is that the initiation of autoimmune diabetes and its etiopathogenesis is much more complex and might involve aberrant recognition of self-nucleic acids at a very early stage. Recent findings from several groups have suggested the role of self-nucleic acids in elevating IFN induced responses by involving several PRRs in various autoimmune disorders including T1D. We would further like to propose that recognition of these self-nucleic acids by various innate immune cell subsets may have a similar outcome as in other autoimmune diseases, like SLE and psoriasis, where DAMPs like self-nucleic acids play a crucial role in the precipitation of the disease. However, despite this growing knowledge, further insights are required on the role of various nucleic acids and their sensors particularly in the context of the regulation of their downstream signaling mediators during the pathogenesis of T1D. Thus, it becomes necessary to search for novel inhibitors or receptor antagonists as a way of modulating dysregulated nucleic acid sensing, which might be useful in preventing or delaying the progression of T1D and similar autoimmune diseases.

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REVIEW

Interactions between diabetes and COVID-19: A narrative review

Sophia Sabri, Olivier Bourron, Franck Phan, Lee S Nguyen

ORCID number: Sophia Sabri 0000-0002-6014-6261; Olivier Bourron 0000-0001-7348-7161; Franck Phan 0000-0002-1412-9687; Lee S Nguyen 0000-0002-6014-6269.

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Sophia Sabri, Intensive Care Medicine, CMC Ambroise Paré, Neuilly-Sur-Seine 92200, France

Olivier Bourron, Franck Phan, Sorbonne Université Médecine; Assistance publique Hôpitaux de Paris (APHP), Service de Diabétologie, Hôpital Pitié-Salpêtrière; INSERM UMRS_1138, Centre de recherche des Cordeliers; Institute of CArdiometabolisme and Nutrition (ICAN), Paris 75013, France

Lee S Nguyen, Research and Innovation, RICAP, CMC Ambroise Paré, Neuilly-Sur-Seine 92200, France

Corresponding author: Lee S Nguyen, MD, PhD, Doctor, Senior Researcher, Research and Innovation, RICAP, CMC Ambroise Paré, 25-27 Boulevard Victor Hugo, Neuilly-Sur-Seine 92200, France. nguyen.lee@icloud.com

Abstract

Diabetes, whether due to pancreatic beta cells insufficiency or peripheral resistance to insulin, has been suggested as a risk factor of developing severe acute respiratory disease coronavirus-2 (SARS-CoV-2) infections. Indeed, diabetes has been associated with a higher risk of infections and higher risk of developing severe forms of coronavirus disease 2019 (COVID-19) related pneumonia. Diabetic patients often present associated comorbidities such as obesity, hypertension and cardiovascular diseases, and complications of diabetes, including chronic kidney disease, vasculopathy and relative immune dysfunction, all of which make them more susceptible to infectious complications. Moreover, they often present low-grade inflammation with increased circulating interleukin levels, endothelial susceptibility to inflammation and dysfunction, and finally, hyperglycemia, which increases this risk. Additionally, corticosteroids, which count among the few medications which showed benefit on survival and mechanical ventilation requirement in COVID-19 pneumonia in large randomized controlled trials, are associated to new onsets of diabetes, and metabolic disorders in patients with previous history of diabetes. Finally, SARS-CoV-2 *via* the alternate effects of the renin-angiotensin system, mediated by the angiotensin-converting-enzyme 2, was also associated with insulin resistance in key tissues involved in glucose homeostasis, such as liver, skeletal muscles, and adipose tissue; and also, with impaired insulin secretion by pancreatic β-cells. In this work, we reviewed all elements which may help understand how diabetes affects patients with COVID-19, how treatments affect outcomes in patients with COVID-19, how they may cause new onsets of diabetes, and finally review how SARS-CoV-2 may inherently be a risk factor of developing diabetes, through immune-mediated diabetogenic mechanisms.

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Core Tip: Diabetes features complex interactions with the severe acute respiratory disease coronavirus-2 (SARS-CoV-2). Diabetic patients are at higher risk of severe infections. They often present associated comorbidities such as obesity, hypertension and cardiovascular diseases, and complications of diabetes, including chronic kidney disease, vasculopathy and relative immune dysfunction. Additionally, corticosteroids, which count among the few medications which showed benefit on survival are associated to new onsets of diabetes, and metabolic disorders in patients with previous history of diabetes. Finally, SARS-CoV-2 *via* the alternate effects of the renin-angiotensin system, mediated by the angiotensin-converting-enzyme 2, was also associated with insulin resistance.

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INTRODUCTION

Preexisting diabetes and coronavirus disease 2019

Preexisting diabetes is associated with poor outcomes of coronavirus disease 2019: There is a set of arguments to say that patients with diabetes are at high risk of severe form of coronavirus disease 2019 (COVID-19) pneumonia. Quickly, at the start of the epidemic, reports from China and Italy suggested that diabetes rate ratio was two or three-fold in patients with more severe than in those with less severe infections[1-5]. Patients with diabetes and COVID-19 pneumonia were more likely to require intensive care, present organ failure, hypercoagulability state with increased levels of inflammatory factors[1,3,6]. In terms of absolute risk, three people with diabetes in every 1000 developed fatal or critical care unit-treated COVID-19[7]. In the French COVID CORONADO cohort, only 50% of the 2796 patients with diabetes were discharged and 20% died within 28 d after hospitalization, reflecting the severity of the disease in the diabetic population[8]. In other countries, mortality rate of patients with diabetes and COVID-19 during the first wave was between 11% and 33%[9-11]. In a retrospective study of 7337 cases of COVID-19 in China, 952 patients with pre-existing type 2 diabetes (T2D) required more medical interventions and presented multiple organ failure than non-diabetic individuals[6]. In this study, T2D was significantly associated with the incidence of acute respiratory distress syndrome (ARDS), septic shock, and acute kidney injury with respective adjusted hazard ratios (HR) of 1.44 (95%CI: 1.20-1.73), 1.95 (95%CI: 1.18-3.20) and 3.01 (95%CI: 1.94-4.68). These findings explain the high mortality rate observed in this population. In Italy, for example, a third of people who died from COVID during the first wave were diabetic[12]. In March 28th, 2020, 2112 deaths from confirmed COVID-19 cases were reported to CDC and diabetes was one of the more frequently reported conditions among all cases. Indeed, a third of the patients hospitalized in intensive care had diabetes[13]. On the other hand, the prevalence of diabetes in non-hospitalized patients among COVID-19 patients was 6%, less than the prevalence of diabetes among United States adults estimated to 10.1% [14]. This may suggest that diabetes is more a risk factor of severity in case of COVID-19 than a risk factor of COVID-19 itself. Data from New York hospitals also found a significantly increased risk of severe forms associated with diabetes, which however disappeared after adjusting for potential confounding factors[15-17]. All data converge to say that diabetes is associated with severe forms of COVID-19, but other factors may impact this association. Diabetic patients constitute a very heterogeneous population, in terms of type of diabetes, disease duration, quality of glycemic control, presence of diabetic complications, antidiabetic treatment used, presence of comorbidities such as obesity, hypertension, dyslipidemia, tobacco and cardiovascular diseases. Yet, only

few studies accounted for these potential confounders.

Are patients with diabetes at risk of severe form of COVID-19?

Impact of age in morbi-mortality of COVID-19 in patients with diabetes: As T2D is more prevalent in elderly patients, whether it is a risk factor of COVID-19 independent from age is currently unknown. Cohort studies in COVID-19 patients which focused on patients with T2D, yielded a mean age between 62 (55-68) in China, and 69.8 ± 13.0 in the French nationwide multicenter CORONADO study[8,18]. The latter study aimed to determine predictors of discharge from hospital and death within 28-d after hospital admission in patients with diabetes and COVID-19. After multiple adjustments, older age and history of microvascular complications were associated were most associated with poor outcomes.

In a systematic review and meta-analysis which included observational studies and investigated risk phenotypes of diabetes and association with COVID-19 severity and deaths, older age (> 65 years) was associated with a 3.49 higher relative risk of COVID-19-related death (95%CI: 1.82-6.69)[19]. Several studies have also shown the absence of excess mortality in young or middle aged patients with type 1 diabetes (T1D)[8,20].

Impact of diabetic complications in morbi-mortality of COVID-19: As the organs affected by COVID-19 are the same affected by diabetes, a relevant question which quickly rose was whether diabetes-induced organ injuries impacted COVID-19 severity. In the French CORONADO study, microvascular complications were associated with a greater risk of death[8]. In a Scottish register study, microvascular complications such as retinopathy and nephropathy were also significantly associated with developing fatal or critical care unit-treated COVID-19[7]. In a population-based cohort study of people with diabetes, increased COVID-19-related mortality was associated with cardiovascular and renal complications of diabetes[21]. Diabetic patients with related chronic kidney disease were even more at risk, in a meta-analysis, with a relative risk of COVID-19-related mortality of 2.53 (95%CI: 0.93-6.88)[19].

Impact of type of diabetes and prognosis of COVID-19: Data about T1D are scarce and contradictory due to the heterogeneous study populations and the presence of many limitations. The French CORONADO study showed a low prevalence of T1D among patients with diabetes hospitalized for COVID-19, 2.1%, lower than that expected in the general population. Data also suggested a lower risk of severe prognosis in patients hospitalized with T1D and COVID-19 than in those with T2D, with half the risk of death by day 7. In a Belgian cohort study, risk of hospitalization was similar in 2336 patients with T1D, compared to 15239 normoglycemic individuals (0.21% *vs* 0.17%)[20]. In a large British population cohort study, including 61 million individuals, showed that patients with T1D (n = 263830; 0.4%) presented an increased risk of in-hospital death due to COVID-19 compared with those without known diabetes [OR: 3.50 (95%CI: 3.15–3.89)][22]. In this study, mortality was higher in T1D than in T2D patients. Even then, age represented a risk factor in T1D patients. In the Belgian cohort, those hospitalized for COVID-19 were older [66 years (58-80) *vs* 49 years (35-61), P = 0.010]. Moreover, similarly as in the British study, in the French CORONADO study, no deaths in young patients with T1D (less than 50-55 years old) was reported. In addition, children and adolescents with T1D showed similar risk of infection and subsequent mortality than those without T1D in several cohorts, which emphasize this age-related risk of being infected and developing severe forms of COVID-19 pneumonia in patients with T1D[23,24].

Obesity, an added risk factor of COVID-19 severity: Obesity is highly prevalent in patients hospitalized for COVID-19 and has also been identified as an independent risk factor for the severity of the disease[25]. Obesity and diabetes, especially T2D, are two commonly associated diseases that are supported by epidemiological as well as genetic studies. One study assessed the relationship between obesity classes and COVID-19 prognosis in patients with T2D. Among 1965 patients with T2D, intubation for mechanical ventilation and death were significantly and independently increased in overweight patients [OR: 1.65 (95%CI: 1.05-2.59)], in patients with class I obesity [OR: 1.93 (95%CI: 1.19-3.14)] and class II/III obesity [OR: 1.98 (95%CI: 1.11-3.52)]. In a prospective, community-based, cohort study among 6910695 individuals, a linear increase in risk of severe COVID-19 leading to admission to hospital and death was observed. This risk increase was superior to that expected to diabetes only. The relative risk due to increasing body mass index was greater in people younger than 40 years and African origins[26]. Hence, T2D combined with obesity may be a synergic risk factor of severe COVID-19 pneumonia. Yet it has to be noted that obesity does not

impact mortality as much in in elderly patients[27].

Influence of glycemic control on the prognosis of COVID-19: A key question is the role of hyperglycemia in patients with diabetes and COVID-19. This question must be analyzed differently depending on whether one considers the time before hospitalization, on admission or during the hospitalization phase. Results on glycemic control before hospitalization are contradictory. A major result of the CORONADO study is that glycemic control prior to hospitalization, assessed by the dosage of glycated hemoglobin (HbA1c), does not seem to have a significant impact on the severity of COVID-19 in people with diabetes who are hospitalized. On the contrary, in a population-based cohort study of people with diagnosed diabetes in England, people with T2D had a higher COVID-19-related mortality when HbA1c was superior to 59 mmol/mol (76%) in comparison to an HbA1c in the range 48–53 mmol/mol (65%-70%). In patients with T2D, this between-group difference was more pronounced in those under 70 than in those over 70 years-old. No significant difference was observed for HbA1c and risk of severe form of COVID-19 in patients with T1D[8,21].

On the other hand, association between hyperglycemia at the time of admission and mortality due to COVID-19 is clearer. Sardu *et al*[28] found that a glycaemia > 7.7 mmol/L on admission was associated with outcome in 132 Italian hyperglycemic patients hospitalized for COVID-19 pneumonia. In a Chinese retrospective multicenter study including hospitalized patients with COVID-19, well-controlled glycaemia (defined as a glycemic variability between 3.9 to 10.0 mmol/L) was associated with markedly lower mortality compared to individuals with poorly controlled glycaemia (upper limit of glycemic variability exceeding 10.0 mmol/L) (adjusted HR: 0.14)[6]. In a retrospective study among patients with diabetes and COVID-19 with use of continuous glucose monitoring, both glucose levels of > 8.8 mmol/L and < 3.85 mmol/L were associated with a significantly high risk of composite adverse outcomes of COVID-19 [*i.e.*, need for admission to the intensive care unit (ICU), for mechanical ventilation, for vasopressor-requiring hypotension, multiple organ dysfunction] as well as with a prolonged hospitalization. Higher glycemic variability on admission was also significantly associated with a poorer outcome of COVID-19[29,30]. In contrast, mean sensor glucose level was not significantly associated with morbi-mortality[30].

While hyperglycemia on admission was associated with severe outcomes, question of causality remains elusive. Although Sardu *et al*[28] showed that the greater the decrease in blood glucose the better the outcomes were, in their observational study, causal relationship between correction of hyperglycemia and better prognosis could not be ascertained. In other settings than COVID-19, glycaemia on admission may be used as a biomarker to identify patients at higher risk of severe pneumonia[31,32]. Hence, randomized controlled studies are still needed to definitely answer this causality question.

What is the link between diabetes and the risk of severe form of COVID-19?

There are many hypotheses about the mechanism of how diabetes affects COVID-19 course.

Hyperglycaemia and diabetes are associated with higher risk of infectious diseases: Infectious diseases, including pneumonia are leading causes of death in people with diabetes[33-35]. Specifically, diabetes was previously proven a major risk factor of mortality related to other viruses than severe acute respiratory disease coronavirus-2 (SARS-CoV-2), such as influenza A (H1N1) influenzae or the Middle East respiratory syndrome-related coronavirus (MERS-CoV)[36,37]. Mechanisms of susceptibility towards severe viral infections include neutrophil dysfunction and disturbance in the adaptative immune response in hyperglycemic environment[38-40]. As a matter of fact, hyperglycemia in itself, was associated with immune system impairment, complement fixation or altered cytokines and chemokines production enhancing SARS-CoV-2 replication[38,41-43]. However, severe forms of COVID-19 pneumonia in patients with hyperglycemia do not appear to result from an impaired humoral response against SARS-CoV-2[44]. These elements may explain the observations of improved prognosis associated with better blood glucose control in hospitalized patients with COVID-19, mentioned before.

Altered immune response and hyperinflammation in patients with diabetes and COVID-19: Histopathologic analysis revealed in fatal COVID-19 patients the presence of massive inflammatory cell infiltration in many organs such as lung, myocardial, liver, brain and nerves, kidney and pancreas[45]. Indeed, cytokines are secreted in

great abundance and increased levels of inflammatory cytokines were associated with increased mortality in patients hospitalized for severe COVID-19 pneumonia. Among them, interleukin-6 (IL-6), also associated with cytokine release syndrome, is usually associated with diabetes, and denotes low-grade inflammation with circulating IL-6 Levels higher than in population without diabetes. Interestingly, study cohorts in patients admitted for severe COVID-19 pneumonia, showed higher levels of inflammation biomarkers including circulating IL-6, in patients with diabetes than other patients[46]. In T2D patients suffering from coronavirus infection, monocytopenia, morphological anomaly of increased monocyte size and $CD8^+$T cells specific lymphopenia were observed[47]. The deregulation of innate and adaptive immune responses could lead to the hyperinflammation observed in severe COVID-19, especially in T2D patients. Visceral adipose tissue that is increased in patients with diabetes could represent a reservoir of cytokines and therefore could also explain the disproportionate inflammatory response observed in patients with diabetes and COVID-19.

Obesity and related disorders associated with the risk of severe forms of COVID-19: COVID-19 pneumonia may deteriorate in obese patients with diabetes due to poor respiratory mechanics. Indeed, when combining obesity and diabetes, patients present weaker respiratory muscle strength, reduced lung volume, increased resistance to the airways, impaired gas exchange, dysregulation of ventilatory control and bronchial dysautonomia[48-50]. Thus, COVID-19 severity could be more severe in case of pre-existing lung damage associated with obesity. Furthermore, in case of T2D and obesity, nonalcoholic fatty liver disease (NAFLD) is highly prevalent, and some data suggest that liver steatosis and higher stages of NAFLD (*i.e.*, non-alcoholic steatohepatitis and liver fibrosis) would be a risk marker for SARS-CoV-2 infection severity[51]. Insulin resistance, a common pathophysiologic trait between obesity and T2D, could explain the increased risk of COVID-19 mortality in diabetes and obesity. Indeed, triglycerides and glucose index was closely associated with the severity and morbidity in COVID-19 patients[52]. In addition, complex interactions can occur between adipose tissue and the immune system[53]. Among them, hyperleptinemia and leptin resistance commonly observed in obesity, are implicated in the increased secretion of pro-inflammatory cytokines that sustain and enhance the inflammatory responses. Interestingly, hyperleptinemia and leptin resistance may aggravate clinical outcomes in infectious diseases, including H1N1 influenzae but also COVID-19[54,55]. Adipose tissue can finally constitute a viral reservoir of SARS-CoV-2, exacerbating the severity of COVID-19 through amplification of immune and cytokine activation[56]. Indeed, SARS-CoV-2 has a high affinity to bind the angiotensin-converting enzyme 2 (ACE2) receptors, highly expressed in adipose tissue. Obese patients have more adipose tissue than lean individuals, resulting in more ACE2 receptors. However, association between obesity and viral load has not been confirmed[57]. In sum, it is difficult to date to distinguish the role of overweight or obesity on the severity of SARS-CoV-2 infections in patients with T2D mellitus (T2DM) and the diabetic state itself.

Thromboembolic risk is increased in people with diabetes and COVID-19: COVID-19 is associated with an increased risk of thromboembolic events. Although, as of yet, there is no evidence of increased risk of such events in patients with diabetes in the setting of COVID-19 infections, several publications reported an increased thromboembolic risk in patients with diabetes. In a large population-based study which included 56158 patients with T2D and 168474 control patients, T2D patients exhibited an increased risk of venous thromboembolism (HR: 1.44, 95%CI: 1.27-1.63). Furthermore, the risks of pulmonary embolism were greater in the patients with T2D than in the controls (HR: 1.52, 95%CI: 1.22-1.90)[58]. Interestingly, hyperglycemia potentiates coagulation, whereas hyperinsulinemia inhibits fibrinolysis, suggesting that T2D patients may be especially vulnerable to prothrombotic events during inflammatory states such as COVID-19[38,59].

Endothelial cell dysfunction is observed in patients with diabetes and COVID-19: Histopathologic studies in patients with COVID-19 revealed evidence of viral presence in endothelial cells, and endothelitis was found in vascular beds of multiple organs such as heart, kidney, lungs, small intestine, and liver[60]. Vascular endothelial dysfunction seem to contribute to the pathophysiology of SARS-CoV-2 infection, by causing inflammatory cell infiltration, endothelial cell apoptosis and microvascular prothrombotic effects[61]. Interestingly endothelial dysfunction also features in patients with diabetes[62]. Hence, infection of dysfunctional endothelial cell by SARS-CoV-2 may be additive to that of diabetes.

TREATMENTS, DIABETES AND COVID-19

Impact of antidiabetic treatments on COVID-19 prognosis

The question of the benefit/risk balance of anti-diabetic treatments in patients suffering from COVID-19 quickly arose during the first half of 2020. The benefits and risks of each antidiabetic treatment as well as the recommendation for their use during COVID-19 are summarized in Table 1.

Insulin: Insulin is often the recommended first-line anti-diabetic treatment in severe sepsis, especially if there is associated organ failure. Insulin infusion may be effective to achieve glycemic targets and improve outcomes in patients with COVID-19[28]. Although study design precluded causality analyses, patients with hyperglycemia treated with insulin infusion showed lower risk of severe disease than patients without insulin infusion. Meanwhile, other studies showed opposite results, with significant association between insulin treatment and a poorer prognosis of COVID-19 [8,63,64]. Remarkably, the association between insulin use and mortality was independent of patients' age. Nevertheless, the worse outcome in patients under insulin may be related to a more severe and complicated overall state rather than a treatment effect. This treatment is also more frequently associated with glycemic variability, which has been associated with severe forms of COVID-19. Insulin treatment remains, until now, the standard treatment in diabetic patients with severe forms of COVID-19.

Dipeptidyl peptidase-4 inhibitors: Dipeptidyl peptidase-4 (DPP4) inhibitors modify the biological activity of substrates involved in the immune response to the infection and therefore could have potential benefit or harm in COVID-19 course. However, evidence from clinical trials on the association between the use of DPP4 inhibitors and the risk of community-acquired pneumonia in T2D patients did not show any increased risk[65]. Although ACE2 represents the main receptor, DPP4 might also bind to SARS-CoV-2[66]. Hence, DPP4 inhibition may play a role in antagonizing the DPP4/CD26, which interacts with the S1 domain of the viral spike glycoprotein, protein by which SARS-CoV-2 attaches to the ACE-2 receptor expressed on the cells surface[67]. Some studies showed better outcome in COVID-19 patients taking DDP-4 inhibitors, with less severe pneumonia and lower mortality risk[64]. However, in a large register study, covering almost the entire population of patients with type 2 diabetes and COVID in England, DPP-4 inhibitors had a higher risk of COVID-19 related mortality[63]. However, here again the existence of confounding bias doesn't allow to conclude to a causal effect. Finally, a propensity score analysis from the CORONADO study concluded that use of DPP-4 inhibitors during the COVID-19 pandemic was safe and that they should not be discontinued[68].

Sodium/glucose cotransporter 2 inhibitors: Given the risk of ketoacidosis, especially in severe sepsis, some have recommended not to prescribe sodium/glucose cotransporter 2 inhibitors (SGLT-2is) in patients with COVID-19, since SGLT-2is are associated with an increased risk of ketoacidosis[69]. However, SGLT-2is could impact many processes dysregulated during COVID-19. For example, SGLT-2is reduced, in T2D patients, infiltration of inflammatory cells into arterial plaques and decreased the mRNA expression levels of some cytokines and chemokines, such as TNF, IL-6 and monocyte chemoattractant protein 1[70,71]. This pharmacological class also features significant cardiovascular and reno-protective benefits in cardiometabolic disease, and may provide similar organ protection in COVID-19. Khunti *et al*[63] showed, in a register study, that SGLT-2is are associated with a significant 18% mortality reduction due to COVID-19. DARE-19, a randomized, double-blind, placebo-controlled trial in 1250 patients is aiming to evaluate the safety and efficacy of dapagliflozin in addition to standard of care therapy in hospitalized patients with COVID-19 and high risk of severe form including T2D[72]. The full DARE-19 trial results will be presented shortly and could answer to the question of the usefulness of iSGLT-2 in COVID-19.

GLP-1 analogues: GLP-1 analogues (GLP-1a) could represent a good therapeutic alternative to treat T2D patients. First, targeting GLP-1 axis could improve many pathways dysregulated during COVID-19. Exendin-4 can reduce inflammation, macrophages activation and monocyte adhesion to endothelial cells and improve endothelial function[73,74]. Second, GLP-1a could ameliorate lung injury in animal models[75]. Furthermore, GLP-1a have cardiovascular and reno-protective properties that could be beneficial during SARS-CoV-2 infection. In the other hand, GLP-1a have been associated with increased ACE2 expression in lungs and heart tissue suggesting of possible helpful and harmful effects in COVID-19[76]. Khunti *et al*[63] have shown,

Table 1 Use of antidiabetic medications in patients with type 2 diabetes and coronavirus disease 2019

	Insulin	Sulfonylurea	Metformin	DPP-4 inhibitors	SGLT-2is	GLP-1a
Benefits	Guarantee of achieving glycemic control; Cardiovascular neutrality; Possible use in multivisceral failure	Cardiovascular neutrality (demonstrated only with Glimepiride)	Probable cardiovascular benefit; No risk of hypoglycemia improvement of inflammation and endothelial dysfunction	Cardiovascular neutrality; Possible use in severe renal impairment and hypoxia; Possible inhibitory role on the entry of the virus into the cell; No risk of hypoglycemia	Proved cardiovascular and renal protective benefits; No risk of hypoglycemia Improvement of inflammation	Proved cardiovascular and renal protective benefits; Possibility of use up to the stage of severe renal failure; No risk of hypoglycemia; Improvement of inflammation and endothelial dysfunction
Risks	Increased glycaemic variability hypoglycaemic risks	Hypoglycaemic risk, contraindication in case of severe liver and renal failure	Multiple contraindications (hypoxia, severe renal failure, severe heart failure, severe liver failure); Risk of lactic acidosis especially in severe renal failure	Possible dysregulation of T cell function and T cell mediated inflammatory and immune responses	Reduced efficacy in moderate to severe renal impairment; Risk of ketoacidosis, especially in severe sepsis Risk of dehydration	Risk of digestive side effects; Risk of worsening undernutrition
Association with severe form of COVID-19 in observational studies	Conflicting results	Lower risk of severe form of COVID-19 or neutral association	Lower risk of severe form of COVID-19 or neutral association	Conflicting results	Lower risk of severe form of COVID-19 or neutral association	Neutral association
Medication use and severity of COVID-19 infection	Possibility of use at all stages of the disease and particularly in severe forms, especially recommended if blood sugar level is over 10-11 mM	Possible use up to moderate forms in the absence of severe renal and liver failure	Recommended use up to moderate forms in the absence of contraindications	Possible use up to moderate forms	Possible use up to moderate forms in the absence of moderate to severe renal failure	Possible use up to moderate forms

COVID-19: Coronavirus disease 2019; DPP-4: Dipepetidyl peptidase-4; SGLT-2is: Sodium-glucose cotransporter-2 inhibitors; GLP-1a: Glucagon like peptide-1 analogues.

in a register study, that GLP-1a had a neutral effect for COVID-19-related death, as observed in the CORONADO study[8]. Although there is insufficient data to support the use of GLP-1a instead of insulin in patients with T2D and COVID-19, there is no evidence to discontinue them.

Sulfonylureas: Sulfonylureas are not associated with higher risk of COVID-19 mortality or are even sometimes associated with a lower risk[63]. However, no conclusion can be drawn since several biases exist in these studies. Indeed, use of sulfonylureas is limited in older and frailty people, in view of an increased risk of hypoglycemia.

Metformin: Many studies indicate that chronic metformin usage may have beneficial effects on COVID-19 with pre-existing T2D[19,63,77,78]. The findings of these studies could be related at least in part to confounding biases. Indeed, metformin is used early in the disease course of T2D, whereas other treatments such as insulin are initiated later or in case of contraindication to metformin such as renal failure. But surprisingly, benefits of metformin on COVID-19 outcomes occurred in spite of an apparently greater severity on admission compared with non-users[77]. Metformin could have beneficial effects on COVID-19 prognosis because of their protective properties on many cell types (*e.g.*, endothelial cells, neurons and glial and cells, cardiomyocytes, hepatocytes, macrophages)[79]. Activation of adenosine monophosphate-activated protein kinase pathways by metformin could also affect the expression of ACE2, the receptor for SARS-CoV-2[80]. Interestingly, one study have shown that metformin benefits would be greater in female patients with diabetes and COVID-19[81]. Besides COVID-19 setting, metformin use is also associated with a reduction in mortality from sepsis in diabetic patients hospitalized in intensive care units[82]. Although there is no direct evidence for a protective role of metformin in SARS-CoV-2 infection, some elements plead in favor of maintaining this treatment in the absence of contrain-

dication and in particular acute renal failure or hypoxia.

Treatments against COVID-19: Corticosteroids

COVID-19 pneumonia may various presentations, from the most benign cough to the most severe ARDS requiring venovenous extracorporeal membranous oxygenation. The pathophysiological features of severe cases of COVID-19 are damages of the alveolii, inflammatory infiltrates, and microvascular circulation disorder leading to thrombosis. As previously stated, inflammatory organ injury may also, and multiples therapeutics have been suggested to decrease inflammatory organ injury but the effect of glucocorticoids has been debated.

Since the publication of results of the RECOVERY trial[83], all guidelines concur towards the use of corticosteroids in COVID-19 pneumonia: In patients hospitalized with COVID-19, use of dexamethasone resulted in lower 28-d mortality among those who were receiving either invasive mechanical ventilation or oxygen alone at randomization. Study protocol specified 6 mg of intravenous dexamethasone per day for 10 d. However, many patients are now treated at home, and French high council for public health recommended as alternative the use of methylprednisolone, 32 mg per day, or prednisone, 40 mg per day, or at very least the hydrocortisone at the dose of 160 mg for days (with decrease in 3 or 4 d), based on glucocorticoid equivalence, although results were less conclusive by oral route.

In a large meta-analysis, patients randomized to receive systemic dexamethasone, hydrocortisone, or methylprednisolone were compared to those who received usual care or placebo[84]. Primary outcome was all-cause mortality at 28 d. This analysis included 1703 patients, with 5 trials which reported mortality at 28 d, 1 trial at 21 d, and 1 trial at 30 d. There were 222 deaths among 678 patients randomized to corticosteroids and 425 deaths among 1025 patients randomized to usual care or placebo [odds-ratio = 0.66 (95%CI: 0.53-0.82), $P < 0.001$], in agreement with RECOVERY. Hereafter, medical community acknowledged the usefulness of corticosteroids in treating COVID-19 patients. Yet, corticosteroids are associated with well-known side effects, one of which being steroid-induced diabetes, for which other risk factors may increase this risk of adverse reaction. Meanwhile, means to prevent steroid-induced diabetes also exist.

Steroid-induced diabetes mellitus

This entity is defined as an abnormal increase in blood glucose due to the treatment, in patients with or without previous history of diabetes. Thresholds are for 8-h fasting glucose: Above 7.0 mmol/L; after 2-h post-75 g oral glucose tolerance test: Above 11.1 mmol/L (2 g/L); HbA1c above 6.5%; or in symptomatic patients, a random plasma glucose above 11.1 mmol/L (2 g/L)[85]. Prevalence is estimated to between 18.6% and 25% of patients who use of corticosteroids daily[86]. In a meta-analysis which aggregated 13 studies and included 34907 non-diabetic patients treated with glucocorticoids, the incidence of hyperglycemia was 32.3% and that of diabetes was 18.6%[87].

Mechanisms are plural and mostly feature beta cell dysfunction and insulin resistance. Beta cell dysfunction participates to insulin insufficiency due to decreased systemic release, and decreased sensitivity to glucose. Insulin resistance occurs in the liver, skeletal muscle, and fat cells, leading to decreased intracellular signals mediated by insulin. From a molecular point of view, glucocorticoids use schematically leads to a decrease in phosphorylation of the protein kinase B (PKB), which in turn leads to a decrease in activity of the glucose transporters GLUT4, which are less translocated to the cell surface, hence, lead to insulin resistance and decrease in glucose uptake, particularly at the muscle and adipose tissue level[88]. Besides, glucocorticoids induce an upregulation of the enzyme phosphoenolpyruvate carboxykinase (PEPCK) activity in the liver, while simultaneously downregulating PEPCK activity in adipose tissue. Circulating free fatty acids increase, which leads to insulin resistance and gluconeogenesis.

While the effects of glucocorticoids widely differ based on patients who use them, even in healthy individuals, they may have a significant impact on metabolism, in animal models[89], and even in healthy people[90]. In a study which focused on metabolomic profiling in 20 healthy men, 214 plasmatic metabolites were analyzed before and after the administration of dexamethasone 4 mg. Overall, 150 of 214 metabolites were significantly altered even after a single dose of dexamethasone. All main energy pathways, including glycolysis, Krebs cycle, urea cycle and lipids, fatty acids and amino acids were altered with an expected inter-individual variability.

Given that glucocorticoids may have that much of an impact even in healthy individuals, patients more at risk of developing diabetes such as elderly patients, overweight or already insulin resistant patients may be even more impacted by the smallest dosages of glucocorticoids. In addition to usual risk factors of developing diabetes, other comorbidities are at risk of developing subsequent steroid-induced diabetes. Specifically, rheumatic disorders and patients afflicted with chronic kidney disease, treated by glucocorticoids may be at higher risk. In a retrospective study which included 128 non-diabetic patients with either rheumatic or renal disease who started glucocorticoids, 84 (65%) developed diabetes, much higher that the incidence observed in the overall population[91]. Independent variables associated with incidence of diabetes included age 65 years, HbA1c level 6% and glomerular filtration rate below 40 mL/min/1.73m^2. Interestingly, dosage did not influence risk of developing diabetes.

While dexamethasone treatment indicated during COVID-19 treatment is relatively short (10 d), adverse effects may occur. In-vitro, beta cell function was studied under three regimens of dexamethasone (0.1, 0.5, and 1.0 mg/kg) for 5 d. In the first group (0.1 mg/kg) beta cell function increased to satisfy insulin demand. In the second group (0.5 mg/kg), beta cell proliferation increased, associated with hyperinsulinemia but not hyperglycemia. Finally, the last group (1.0 mg/kg) presented hyperglycemia and hyperinsulinemia, and a major increase in beta-cell proliferation and size[92]. In-vivo, in 6 healthy men, a single-dose of 75 mg prednisolone did not change fasting plasma glucose or insulin, but decreased oral glucose insulin sensitivity[93]. On day 2 beta cells recovered, as evidenced by an increase in fasting insulin secretion. The same study also looked at the impact of a 2-wk exposure of prednisolone in 33 healthy men, the treatment increased the fasting plasma glucose, decreased the index of insulin resistance, and decreased the index of insulin sensitivity. These elements showed that prednisolone impairs beta cell function in healthy subjects, both in acute and 2-wk exposure, meaning that steroids induce insulin resistance but also beta cell dysfunction, even for these short periods of administration.

Interestingly, in RECOVERY trial, only few severe adverse events related to dexamethasone administration were reported, yet, among 4 severe adverse events reported, 2 (50%) included severe hyperglycemia[83]. Only time will allow to assess the incidence of diabetes in patients treated with dexamethasone, as compared to control treatment. Moreover, the influence of COVID-19 itself on the risk of developing diabetes needs to be accounted for.

COVID-19 AS A RISK FACTOR OF DIABETES

SARS-CoV-2 and diabetes

Infections, including COVID-19, may induce hyperglycemia in people without a previous diagnosis of diabetes. In fact, patients may present with stress hyperglycemia and thus, surpass the threshold only in the context of SARS-CoV-2 infection. Thus, hyperglycemia *per se* is not specific to COVID-19, especially since acute diabetes was commonly observed during SARS-CoV-1 epidemic among patients without prior history of diabetes and before the use of glucocorticoids[37]. However, the question has been raised as to whether SARS-CoV-2 can cause diabetes since new diabetes onset have been reported in numerous case reports simultaneously with acute SARS-CoV-2 infection. Although a meta-analysis of 8 studies including more than 3700 patients hospitalized for COVID-19 revealed a pooled incidence of 14.4% for new onset diabetes[94], mechanisms by which SARS-CoV-2 may induce diabetes remain unclear. Traditionally, 2 components are known to feature in diabetes pathogenesis: (1) Insulin resistance in the key tissues involved in glucose homeostasis, *i.e.*, liver, skeletal muscles, and adipose tissue; and (2) Impaired insulin secretion by pancreatic β-cells.

SARS-CoV-2 and ACE2

Structural evidence reported that ACE2 was the receptor of SARS-CoV-2. Viral infection of host cells occurs through viral spike protein and ACE2 receptor. SARS-CoV-2 entrance into host cells then induces ACE2 internalization and shedding, leading to a down-regulation of ACE2.

Of note, ACE2 belongs to the renin-angiotensin system (RAS). Briefly, angiotensinogen, produced by the liver, is cleaved by renin to form angiotensin-I which is then catalyzed by ACE to produce angiotensin-II (Ang II). ACE2 is a homologue of ACE that can hydrolyze Ang II to Ang-(1-7), whose reported effects include vasodilatation, anti-fibrosis, and anti-inflammation (these elements are

summarized in Figure 1). As Ang II is associated with insulin resistance[95], a major component of T2DM, disturbance of ACE2 activity by SARS-CoV-2 in glucose homeostasis key-tissues may induce acute hyperglycemia. In addition, ACE2 expression was also reported in the endocrine pancreas suggesting that SARS-CoV-2 may cause beta cell damage inducing diabetes through insulin secretion deficiency [96], but also in exocrine pancreatic cells[97]. Therefore, dysregulation of ACE2 activity caused by COVID-19 infection could lead to diabetes through several mechanisms.

SARS-CoV-2/ACE2 and insulin resistance-induced diabetes

One of the most obvious mechanism by which SARS-CoV-2 induces insulin resistance is through systemic inflammation. Indeed, any inflammatory state can cause an insulin resistance leading to an increase hepatic glucose production through increased counter-regulatory hormones, cytokine and lipid release, and direct hepatocyte injury, irrespective of specific potential effects of SARS-CoV-2 on ACE2 activity and its repercussions on glucose homeostasis.

Then, since ACE2 is expressed in liver, skeletal muscles and adipose tissue, a disturbance in ACE2/Ang-(1-7) activity could lead to a glucose homeostasis disorder. Further support to this hypothesis comes from the fact that SARS-CoV-2 viral particles were not only identified in the cytoplasm of hepatocytes inducing a massive hepatic apoptosis[98], but were also involved in myositis occurrence[99]. Moreover, the loss of ACE2 gene in mice leads to hepatic fibrosis and impaired glucose homeostasis through an elevated hepatic reactive oxygen species level, an increased oxidative stress and inflammation in liver leading to an impairment of insulin signaling[100]. In adipose tissue, ACE2 deficiency worsens inflammation in response to a diet-induced obesity in mice[101]. Conversely, overexpression of ACE2 or Ang-(1-7) administration improves these metabolic disorders *i.e.,* glycemic control and insulin sensitivity[102-104]. Indeed, mechanistically, Ang-(1-7) rescues insulin signaling pathway by stimulating PKB phosphorylation, a main mediator of insulin signaling pathway, what will then activate the downstream glycogen synthase kinase-3β in liver and skeletal muscles resulting in a decrease of glycaemia through glycogen storage[105], in several murine models of diet-induced insulin resistance such as high-fat diet fed mice or in fructose-fed rats[106,107]. In adipose tissue, activation of ACE2/Ang-(1-7) prevents inflammation and oxidative stress induced by a high-fat diet and increases glucose uptake and adiponectin level[108-110], while its disturbance results in a lower insulin-dependent glucose uptake and adiponectin secretion[111].

Besides the effects of the inhibition of the above mentioned alternate effects of the RAS, in the context of metabolic diseases such as obesity, T2DM or nonalcoholic fatty liver disease, plasmatic Ang II is positively correlated with body weight and is associated with insulin resistance, suggesting that ACE/Ang II activity is upregulated in those metabolic disorders[95]. Besides, on a tissue scale, Ang II was associated with increased insulin resistance through oxidative stress leading to a hepatic fibrosis and cirrhosis, provoking an impairment of insulin signaling[112]. In skeletal muscles, Ang II also induces a decreased glucose uptake and impairs insulin sensitivity[113], while in adipose tissue, it inhibits adiponectin secretion and insulin signaling still through an increased oxidative stress[114]. These elements emphasize the pro-diabetogenic effects of the classical RAS effects.

SARS-CoV-2/ACE2 and insulin secretion deficiency-induced diabetes

In a few COVID-19 human pancreas postmortem examinations, SARS-CoV-2 nucleocapsid protein were detected in pancreatic exocrine cells as well as in endocrine β-cells [115]. Furthermore, the RAS, including ACE2 was also found involved in the pancreatic insulin-producing tissue[116]. However, ACE2 expression by β cells responsible for insulin secretion remains controversial. Indeed, analyses from transcriptional datasets of human islet cells find a very weak expression of ACE2 in beta cells. These analyses are supported by immunohistochemistry of human pancreatic tissues that do not identify ACE2 expression on β cells but rather on ducts and microvascular structures[97], whereas double immunofluorescence labelling in rat pancreas indicates that insulin-containing beta cells abundantly express ACE2[117]. The latter observations suggest that ACE2 would play a role in insulin-containing beta cells and are supported by experiments in ACE2-deficient mice indicating that ACE2 loss aggravates beta cell dedifferentiation and impairs their proliferation, leading to a significant reduction of beta cell mass[118]. Similarly, in a genetic murine model of obesity and diabetes, ACE2 overexpression in pancreas, improved glycemic control through Ang-(1-7), inducing both β-cell proliferation and apoptosis reduction[102]. As expected, similarly as a loss of ACE2, Ang II supplementation in beta cells significantly increased endoplasmic reticulum (ER) stress and inflammation leading to reduced

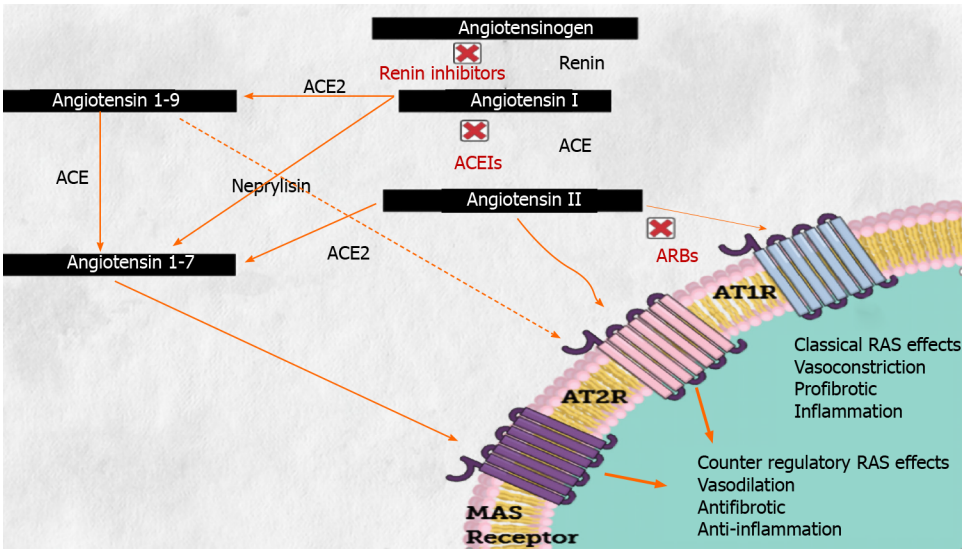

Figure 1 Renin angiotensin system: Classical and counter regulatory pathways. ACE: Angiotensin converting enzyme; ACE 2: Angiotensin converting enzyme type 2; ACEIs: Angiotensin-converting enzyme inhibitors; ARBs: Angiotensin-2 receptor 1 blockers; AT1R: Angiotensin II receptor type 1; AT2R: Angiotensin II receptor type 2; RAS: Renin-angiotensin system. Figure redrawn with permission from the authors (D. Laghlam *et al*).

insulin secretion whereas Ang II receptor blockade in beta cells reduced significantly ER stress and rescues insulin secretion[119].

Moreover, ACE2 was also found on ductal structures and microvasculature of the pancreas, new diabetes onset may be secondary to pancreatitis as SARS-CoV-2 has been isolated in a pancreatic pseudocyst from a patient with acute pancreatitis. However, acute pancreatitis seems to be a very infrequent complication of SARS-CoV-2 infection. Two cohort studies which included 11000 and 63822 patients with COVID-19 respectively, acute pancreatitis prevalence was estimated at 0.27% and 0.07%[120]. Therefore, acute pancreatitis occurrence in patients with COVID-19 is exceedingly rare and its putative mechanism related to direct viral damage of pancreatic cells still need investigations.

Therefore, these findings suggest that ACE2 may play a role in the beta cell insulin secretory response to hyperglycemia and imply that SARS-CoV-2 could penetrate then destroy the insulin-containing beta cells, causing subsequently acute diabetes through insulin secretion deficiency.

SARS-CoV-2 and autoimmune type 1 diabetes

Viral infections, in particular by enteroviruses and coronaviruses, have been widely associated with T1DM pathogenesis[121]. T1DM is characterized by an autoimmune pancreatic β-cells progressive destruction leading to insulin deficiency. Therefore, SARS-CoV-2 could also act as an infectious trigger decompensating and precipitating diabetic ketoacidosis in patients with no history of diabetes as reported in few case reports[122-124], and arising evidence highlight the ability of SARS-CoV-2 to trigger autoimmune disorders[125]. Nonetheless, data remain conflictual on this point. Evidence from an italian cross-sectional study revealed 23% fewer new-onset cases, with more children with new-onset disease presenting in diabetic ketoacidosis during early months of pandemia compared to the same period in 2019 while a multicenter study from the United Kingdom described an 80% increase in new-onset T1DM in children[126]. From a German Diabetes-Prospective Follow-up registry, the rate of new-onset T1DM from March to May 2020 did not differ significantly from rates observed over the previous decade[127]. However, when this study was done, COVID-19 infection incidence rate was relatively low in Germany, and weak effect cannot be excluded. Thus, from these studies, no compelling evidence emerge for a causal role of SARS-CoV-2 in a change of T1DM incidence. Furthermore, it was difficult to differentiate a viral secondary diabetes from a real T1DM as no assay for type 1 diabetes antibodies (GAD, IA2, ZNT8, ICA antibodies) has been performed in those series. More complexly, a few cases of insulin-dependent diabetes with negative antibodies start to emerge suggesting a T1bDM[128]. However, such form of diabetes is particularly widespread among Sub-Saharan African, African-Americans and Hispanic descendants and case reports concern Caucasian and Asian ethnicities suggesting a viral secondary diabetes more than a T1DM or T1bDM. Follow-up

studies on the evolution of anti-diabetic therapy are needed to understand the pathophysiology of SARS-CoV-2-induced diabetes.

In the end, the potential diabetogenic role of SARS-CoV-2 may be more complex than the simple beta cell hosts destruction by the virus through ACE2 expression. New-onset diabetes can result from several pathogenic processes involving pancreatic cell destruction (including exocrine and endocrine cells) through viral or autoimmunity destruction and/or insulin resistance in liver, skeletal muscles, and adipose tissue through disturbance of ACE2/Ang-(1-7) activity.

CONCLUSION

Diabetic patients are heavily impacted by the effects of COVID-19, as they are more at risk of developing severe forms and are more at risk of mortality. While antidiabetic treatments are still under investigation, data do not warrant discontinuation of these treatment in diabetic patients. While corticosteroids count among the few validated medications in severe COVID-19 pneumonia, they expose patients to a hypothetical risk of new onset of diabetes or diabetes deterioration, even though treatment duration is short. Finally, risk of developing diabetes after COVID-19, due to interactions with the angiotensin-converting-enzyme 2 needs to be accounted for when assessing risk of subsequent diabetes in treated patients.

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MINIREVIEWS

Diabetes and gut microbiota

Yue Xi, Peng-Fei Xu

ORCID number: Yue Xi 0000-0003-1265-6624; Peng-Fei Xu 0000-0001-6854-6040.

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Yue Xi, Peng-Fei Xu, Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15261, USA

Peng-Fei Xu, Key Laboratory for Cell Proliferation and Regulation Biology of State Education Ministry, College of Life Sciences, Beijing Normal University, Beijing 100875, China

Corresponding author: Peng-Fei Xu, PhD, Academic Research, Postdoctoral Fellow, Research Associate, Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, 323 Salk Pavilion, 335 Sutherland Drive, Pittsburgh, PA 15261, United States. pex9@pitt.edu

Abstract

The prevalence of diabetes has increased rapidly throughout the world in recent years. Currently, approximately 463 million people are living with diabetes, and the number has tripled over the last two decades. Here, we describe the global epidemiology of diabetes in 2019 and forecast the trends to 2030 and 2045 in China, India, USA, and the globally. The gut microbiota plays a major role in metabolic diseases, especially diabetes. In this review, we describe the interaction between diabetes and gut microbiota in three aspects: probiotics, antidiabetic medication, and diet. Recent findings indicate that probiotics, antidiabetic medications, or dietary interventions treat diabetes by shifting the gut microbiome, particularly by raising beneficial bacteria and reducing harmful bacteria. We conclude that targeting the gut microbiota is becoming a novel therapeutic strategy for diabetes.

Key Words: Diabetes; Gut microbiota; Epidemiology; Probiotics; Anti-diabetic medication; Diet

Core Tip: The current review describes the global epidemiology of diabetes in 2019 and forecasted the trends to 2030 and 2045 in China, India, USA, and globally. This review also summarizes the interaction between diabetes and the gut microbiota in three aspects: probiotics, antidiabetic medications, and diet.

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INTRODUCTION

The global prevalence of diabetes has grown rapidly in recent decades. Diabetes is becoming a serious global health threat, and is one of the top 10 leading causes of death among adults[1]. The etiology and progression of diabetes are commonly driven by genetic and environmental factors. The International Diabetes Federation (IDF) estimates that in 2019 there were 463 million cases of diabetes mellitus worldwide and approximately 4.2 million adults died from diabetes and its complications[2]. It is estimated that approximately 700 million adults will be diagnosed with diabetes by 2045. Diabetes mellitus is a group of metabolic diseases that cause high blood glucose, and primarily includes type 2 diabetes (T2D), type 1 diabetes, prediabetes, and gestational diabetes. T2D is the most common type of diabetes and represents approximately 90% of all diabetes patients worldwide[3].

The gut microbiota is a collective term for the intestinal microbial community, which plays a crucial role in maintaining health and disease pathogenesis. Recently, the gut microbiome has become an emerging research area for diabetes management, as gut dysbiosis directly or indirectly participates in diabetes by affecting host intestinal barrier functions and metabolic homeostasis[4]. Animal and human studies have identified related differences in the composition of the gut microbiota in patients with diabetes[5]. In this review, we describe global trends in diabetes in 2019, predict the trends to 2030 and 2045, and summarize the latest findings regarding the gut microbiota in diabetes.

EPIDEMIOLOGY OF DIABETES

Diabetes is one of the fastest growing global health challenges in the last 40 years, with the number of adults living with diabetes rising from 108 million in 1980 to 463.0 million (368.7–600.6 million) in 2019. This number is projected to reach 578.4 million (456.5–747.6 million) in 2030 and 700.2 million (540.7–904.6 million) in 2045. The global prevalence of adult diabetes increased from 4.7% in 1980 to 8.3% (6.2%–11.8%) in 2019, and is projected to reach 9.2% (6.8%–12.9%) in 2030 and 9.6% (7.1%–13.4%) in 2045[1]. Although the common long-term complications in diabetic patients develop gradually, they could be disabling or even life-threatening over time[6]. Diabetes is a major cause of many diseases, such as eye damage, kidney failure, heart and blood vessel disease, neuropathy, Alzheimer's disease, and lower limb amputation. Global diabetes-related health spending continues to grow rapidly as well. It was 760 billion US dollars in 2019, approximately 10% of total global health spending, and is expected to reach 825 billion US dollars in 2030 and 845 billion in 2045[7].

China and India were the two countries with the highest number of adult diabetic patients in 2019 and are projected to remain so in 2030 and 2045, due to the demographic and socioeconomic status factors. The IDF Diabetes Atlas (9th edition 2019) estimated the number of people with diabetes in China, India, USA, and the world in 2019, and projected that by 2030 and 2045 (Figure 1), the number of adults living with diabetes in China will increase from 116.4 million (108.6–145.7 million) in 2019 to 140.5 million (130.3–172.3 million) in 2030, and 147.2 million (134.7–176.2 million) in 2045. In India, the number of diabetes cases is projected to grow from 77.0 million (62.4–96.4 million) in 2019 to 101.0 million (81.6–125.6 million) in 2030, and 134.2 million (108.5–165.7 million) in 2045. The number of adult diabetes cases in the USA will increase from 31.0 million (26.7–35.8 million) in 2019, to a projected 34.4 million (29.7–39.8 million) in 2030 and 36.0 million (31.0–41.6 million) in 2045. Over the last 40 years, the number of people with diabetes has quadrupled throughout the world. The prevalence of diabetes will increase more rapidly in low-income than in high-income countries in the near future[1]. Unmet medical needs related to diabetes are a growing global public health problem.

INTERACTION BETWEEN DIABETES AND GUT MICROBIOTA

Observational findings from recent epidemiological, physiological and metabolomic

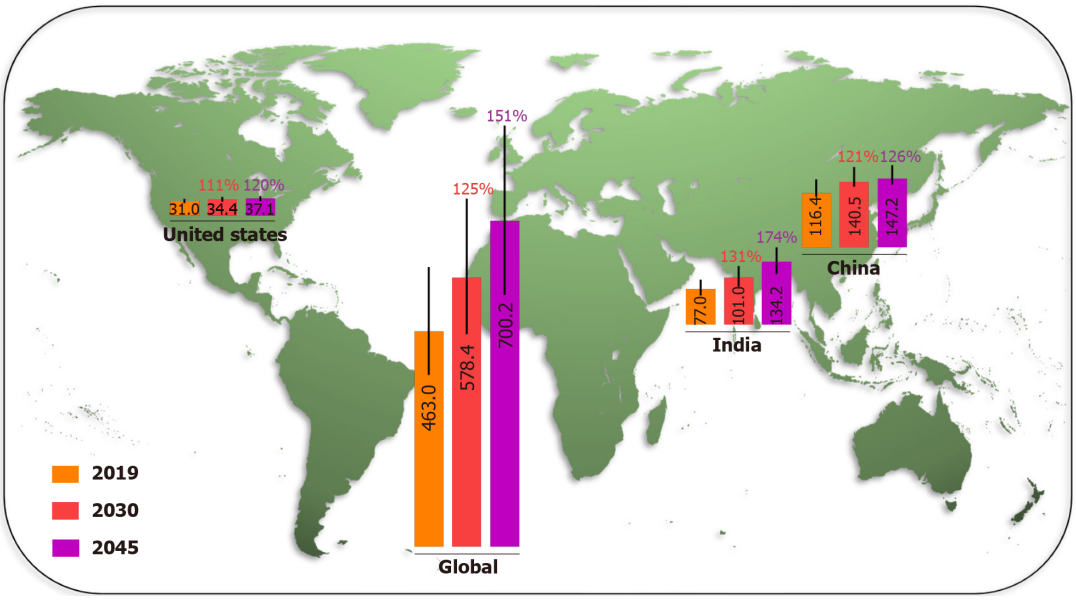

Figure 1 Millions of diabetes cases in 2019 and projections to 2030 and 2045, with projected percentage changes. Data are from the International Diabetes Federation Diabetes Atlas (9th edition 2019).

studies, complemented by cellular and animal experiments and clinical trials, it appears that microbial communities may contribute to the pathogenesis of a variety of common metabolic disorders, including obesity and diabetes, and their complications [3,8]. Although accumulative evidence suggests that the gut microbiota is a factor influencing diabetes, the underlying mechanisms remain unclear. Due to the crosstalk between the gut microbiota and host homeostasis, the gut microbiome is thought to play a crucial role in obesity and associated metabolic dysfunction[9,10]. The gut microbiome has been shown to affect host metabolism, food consumption, body weight, and glucose and lipid homeostasis. Gut dysbiosis or altered microbiota composition has been detected in obesity and diabetes in human and murine models [11]. Treatment with probiotics, antidiabetic medications, or dietary interventions can orchestrate the gut microbiome, leading to increased probiotic bacteria and decreased harmful bacteria, and these changes subsequently contribute to bodyweight loss, suppression of inflammation, and maintenance of glucose homeostasis in the host[12]. Targeting the gut microbiota is developing into a possible therapeutic strategy for diabetes.

Probiotics

Probiotics are living microorganisms that provide health benefits to their host, particularly the digestive system. Probiotics, such as *Akkermansia*, *Bacteroides*, *Bifidobacterium* and *Lactobacillus*, are currently suggested as novel and potential biotherapeutics in the prevention and management of diabetes[13,14]. Oxidative stress is a key player in the development of diabetes and diabetes-related complications[15]. Supplementation with probiotics and also synbiotics could be beneficial for patients diagnosed with diabetes also because these products lower oxidative stress levels[16,17]. Cumulative studies have proven the efficacy of probiotics in the treatment of diabetes by decreasing fasting glucose and insulin levels in animal models and clinical trials[18].

Akkermansia muciniphila is a species of mucin-degrading bacteria recently found in the human gut, and its abundance has been reported to be inversely correlated with obesity, T2D and inflammation[19-22]. Administration of *A. muciniphila* protected against high fat diet (HFD)-induced obesity and insulin resistance by suppressing inflammation and improving gut barrier function. In addition, a purified protein in the outer membrane of *A. muciniphila* called Amuc-1100 could improve metabolic syndrome in obese and diabetic mice through the Toll-like receptor 2 signaling pathway[23]. In human clinical trials, supplementation with *A. muciniphila* compared to the placebo improved insulin sensitivity, reduced insulinemia and plasma total cholesterol, and decreased body weight in overweight/obese insulin-resistant volunteers[24]. In our recent studies, we found that melatonin, a probiotic agent, partially improved insulin resistance by increasing the abundance of *A. muciniphila* in HFD-fed mice[25]. *A. muciniphila* is considered a promising probiotic to improve

diabetes and obesity-associated metabolic disorders.

Bacteroides is a common genus associated with the risk of T2D in patients. However, the role of *Bacteroides* in diabetes is controversial. Some studies have shown that the abundance of *Bacteroides* is inversely associated with diabetes risk[26-30], while others have reported a positive association in different species[31-33]. This inconsistency may be explained by the underlying feedback mechanism of the gut microbiome at different stages of the disease or in different animal models. The ratio of Bacteroidetes to Firmicutes, previously identified as a marker for metabolic diseases, does not seem to be consistently associated with diabetes risk[14]. In animal studies, treatment with *Bacteroides acidifaciens* and *Bacteroides uniformis* prevents obesity and improves insulin susceptibility in diabetic mice[34,35]. These studies suggest that *Bacteroides* may have a beneficial effect on diabetes.

Bifidobacterium, also known as *Lactobacillus bifidus*, is frequently reported in T2D protection studies. *Bifidobacterium* strains are crucial probiotics in the dairy industry, due to their unique function of fermenting carbohydrates *via* the fructose-6-phosphate phosphoketolase pathway[36]. Numerous studies have shown that *Bifidobacterium* has beneficial effects on glucose tolerance in individuals with T2D and diabetic murine models[37-39]. Oral administration of *Bifidobacterium* decreases blood glucose concentration and glycosylated hemoglobin levels, and improves lipid profiles, insulin resistance, and antioxidant indexes, through insulin receptor substrate/phosphoinositide 3-kinase/protein kinase B and kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2 signaling pathway in murine diabetic models[40]. *Bifidobacterium* may be a promising probiotic to treat diabetes.

Lactobacillus is the most commonly used probiotic in industry to control food fermentation, such as yogurt, cheese, wine, and other fermented foods. Studies of the composition of gut microbiota showed some species in this genus were increased in T2D patients, such as *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *Lactobacillus salivarius*, whereas *Lactobacillus amylovorus* was decreased in patients with diabetes[41-43]. Oral supplementation of *Lactobacillus*, such as *Lactobacillus casei*, *Lactobacillus curvatus*, *L. gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus sakei*, exhibited beneficial effects in diabetic mice and individuals with diabetes[44-54]. The antidiabetic mechanism of *Lactobacillus* by inhibiting endotoxin secretion and activating G-protein-coupled receptor 43 pathway has been reported[55]. The combination of *Lactobacillus* and *Bifidobacterium* is widely used in clinical practice to synergistically maintain a healthy digestive tract. Growing evidence supports that probiotics are a safe and effective treatment strategy under certain clinical conditions of diabetes.

Diet

Diet is an essential regulator of the gut microbiome[56]. Interactions between diet and gut microbiota have been reported to affect obesity, insulin resistance, and the chronic inflammatory response of the host[57]. Here, we mainly summarize the roles of diet in the gut microbiome and diabetes.

Diet composition is vital in diabetes development. Diabetes was considered a disease of the rich, because of its high prevalence among the rich who access food more easily, including flour, sugar, fat and meat[58]. It has been shown that diets with high levels of sugar, fat and cholesterol increase the risk of diabetes. These diets cause gut dysbiosis and damage the intestinal mucosal barrier that facilitates the development of diabetes[59,60]. High-fiber diet is a well-known healthy diet with various benefits, such as improving bowel movements, lowering cholesterol, achieving a healthy weight, and controlling blood sugar levels. Dietary fibers consist of cellulose, resistant starch and dextrin, inulin, lignin, pectin, -glucan, and oligosaccharides. They are abundant in whole-grain bread and cereals, legumes, rice, vegetables and fruits, and cannot be completely digested or absorbed by the human digestive system[61,62]. Dietary fibers play an essential role in maintaining the gut microbiota and gut health, as they can be catalyzed and fermented by certain gut microbes and produce beneficial metabolites, such as short-chain fatty acids (SCFAs)[63]. In the gut, approximately 95% of SCFAs are acetate (C2), propionate (C3), and butyrate (C4)[64]. Studies have shown that acetate is mainly produced by bacteria, such as *A. muciniphila*, *Bifidobacterium* spp., *Bacteroides* spp., *Lactobacillus* spp., *Prevotella* spp., *Ruminococcus* spp. and *Streptococcus* spp. through the acetyl-coenzyme A pathway[65,66]. Propionate is mainly produced by *Bacteroides* spp., *Coprococcus catus*, *Dialister* spp., *Megasphaera elsdenii*, *Phascolarctobacterium succinatutens*, *Roseburia inulinivorans*, *Ruminococcus obeum*, *Salmonella* spp. and *Veillonella* spp. through three known pathways, *i.e.*, succinate pathway, acrylate pathway, and propanediol pathway[66,67]. Butyrate is produced primarily in *Anaerostipes caccae*, *Clostridium leptum*, *Coprococcus catus*, *Coprococcus eutactus*, *Eubacterium*

hallii, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and *Roseburia* spp., by enzymatic catalysis, such as butyryl-CoA dehydrogenase, butyryl-CoA transferase, and phosphotransbutyrylase or butyrate kinase[66,68]. SCFAs are critical modulators in pathophysiological events of diabetes. They act directly as histone deacetylase inhibitors and increase protective glucagon-like peptide-1 secretion[69], which decreases blood glucose levels, improves insulin resistance, and suppresses inflammation. Our previous studies have shown that dietary lipid adsorbent montmorillonite regulates intestinal absorption and gut microbiota, such as increasing SCFAs-producing *Blautia* bacteria, thereby preventing obesity and insulin resistance in HFD-fed murine models [70,71]. However, dietary effects on the shift of gut microbiota appear to be temporary [72]. Habitual diets, which have a longer lasting influence on the gut microbiome, may be a viable strategy.

Antidiabetic medications

Metformin is an oral antidiabetic medication. It has been used in the treatment of T2D for > 60 years due to its distinct effects on decreasing glucose production and increasing insulin sensitivity, as well as its safety profile. Metformin originates from *Galega officinalis*, a natural source of galegine[73]. Traditionally, activation of the AMP-activated protein kinase signaling pathway in the liver is thought to be the mechanism of its antidiabetic effects[74]. Recent findings indicate that metformin also orchestrates gut microbiome in mice and humans[43]. Sun *et al*[33] reported that metformin improves hyperglycemia through the gut microbiota-bile acid-intestinal farnesoid X receptor (FXR) axis in T2D patients. FXR is an important target in regulating glucose and lipid homeostasis. Metformin reduces the level of *Bacteroides fragilis* in the gut, leading to an increase in the FXR antagonist, glycoursodeoxycholic acid. Treatment with metformin also increased the abundance of probiotics *A. muciniphila* and SCFA-producing microbiota, such as *Butyrivibrio*, *B. bifidum*, and *Megasphaera* in murine and human studies[31]. Here, we summarize the role of the gut microbiome in the antidiabetic effects of metformin (Figure 2).

Acarbose, an α-glucosidase inhibitor, is an oral prescription medication used to control blood glucose in T2D treatment. Acarbose has been reported to alter the composition of gut microbiota in patients with T2D, in particular increasing the abundance of *Bifidobacterium longum* and decreasing the level of lipopolysaccharides [75]. Vildagliptin, a dipeptidyl peptidase 4 inhibitor, is an oral antihyperglycemic agent that enhances insulin secretion and suppresses glucagon release. Vildagliptin supplementation decreases the level of *Oscillibacter* and increases the proportion of *Lactobacillus* in HFD-induced mouse models[76]. Sitagliptin, another DPP-4 inhibitor, appears to exhibit antidiabetic functions during pregnancy in rats by reducing *Lactobacillus* spp. and increasing *Bifidobacterium* spp.[9,77]. Dapagliflozin, a sodium-glucose cotransporter-2 inhibitor, is a medication used to treat T2D. Treatment with dapagliflozin decreases the ratio of Firmicutes to Bacteroidetes and the abundance of *Oscillospira*, and increases the abundance of *A. muciniphila* in diabetic murine models [78,79]. Thiazolidinediones (TZDs) are a class of oral hypoglycemic agents for the treatment of T2D[80,81]. TZDs function through the activation of the peroxisome proliferator-activated receptor (PPAR) signaling pathway[82,83]. Pioglitazone, a member of TZDs, is widely used to treat T2D. It has been reported that treatment with pioglitazone reduces the α-diversity of the gut microbiota in murine T2D models, which may be one of the mechanisms mediating its antidiabetic function[79]. In our previous studies, Danshensu Bingpian Zhi, a synthetic derivative of danshensu and borneol, is a PPARγ agonist that prevents HFD-induced atherosclerosis, obesity, and insulin resistance in mice in part by reversing intestinal microbiota dysbiosis, such as increasing the ratio of Bacteroidetes to Firmicutes, increasing the level of *Akkermansia*, and reducing the level of the harmful bacterium *Helicobacter marmotae*[84]. These results suggest that gut microbiome is a potential target of many anti-diabetic medications clinically.

Traditional Chinese medicines (TCMs) have a long history of treating diabetes, but their mechanisms are not fully understood. Several studies have suggested that TCMs have multiple therapeutic effects on diabetes, including antioxidation, suppression of inflammation, protection of intestinal mucosal barrier, and inhibition of lipotoxicity, mainly by remodeling the gut microbiota[85]. Berberine, a well-known bioactive alkaloid extracted from TCM *Coptis chinensis*, has been used for the treatment of diarrhea and diabetes. Berberine is useful in diabetes management because its administration is associated with a decrease of obesity indices, such as body mass index and waist circumference[86]. Berberine maintains gut health in rats and humans with diabetes by increasing the abundance of *Bifidobacterium* and *Lactobacillus*, and decreasing the abundance of *Escherichia coli*[87,88]. Gegen Qinlian Decoction can

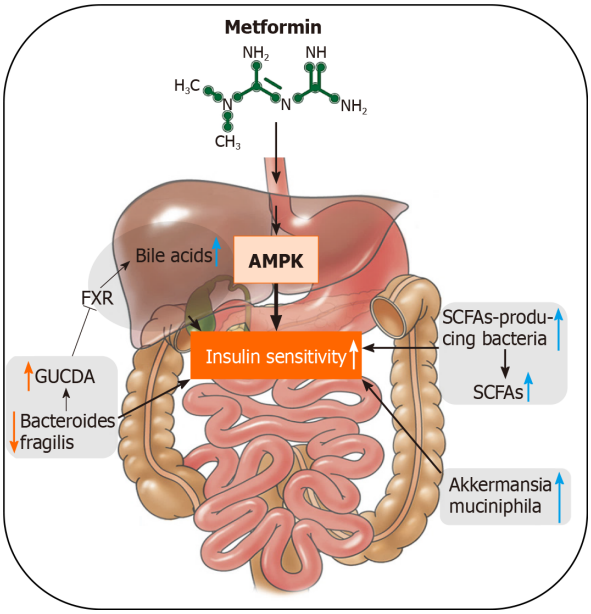

Figure 2 The schematic mechanisms of metformin act through the gut microbiome and the related beneficial effects on diabetes. AMPK: AMP-activated protein kinase; FXR: farnesoid X receptor; GUDCA: glycoursodeoxycholic acid; SCFAs: short-chain fatty acids.

relieve T2D in clinical trials, which is associated with an increase in the level of beneficial bacteria, such as *Faecalibacterium* spp.[89]. In addition, Banxia Xiexin Decoction, Huanglian Jiedu Decoction, and Qijian mixture also have beneficial effects by regulating gut microbiota[85,90,91]. These results suggest that gut microbiota is likely a new direction in elucidating the antidiabetic mechanism of TCMs.

CONCLUSION

Diabetes has become an urgent public health threat, and the growing trend of diabetes cases is expected to continue for the next two decades and beyond. Gut microbiome plays a critical role in health maintenance, and the dysregulation of gut microbiome can contribute to the development and progression of the disease. Here, we summarized the interaction between diabetes and the gut microbiota. Gut dysbiosis is increasingly recognized as a mechanism that induces metabolic diseases. Accumulating studies have shown that the gut microbiome is a key factor in the pathophysiology of diabetes, but research in this area is still in the early stages. Most of the studies have only shown that changes in the composition of the gut microbiota are associated with the progression of metabolic diseases. The exact causal relationship between a specific intestinal bacterium and phenotypic exposure is still not well understood. Further experiments using fecal or bacterial transplantation in germ-free mice and clinical studies are required to obtain a deeper understanding of the roles of individual bacteria in metabolic diseases. The use of metabolomics and transcriptomics to study the gut microbiome is a more effective strategy to understand the role of microbiota in the progression of host disease.

Traditionally, most pharmacological agents used for treatment of diabetes directly regulate the signaling pathways involved in glucose and insulin homeostasis. However, the gut microbiota is becoming an emerging therapeutic target for diabetes. In view of the good performance of herbal agents, particularly TCMs, in regulating gut microbiota, more consideration should be given to the use of medicinal herbs for the treatment of diabetes.

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MINIREVIEWS

Tale of two kinases: Protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II in pre-diabetic cardiomyopathy

Pamela Gaitán-González, Rommel Sánchez-Hernández, José-Antonio Arias-Montaño, Angélica Rueda

ORCID number: Pamela Gaitán-González 0000-0002-8980-1212; Rommel Sánchez-Hernández 0000-0002-4008-4179; José-Antonio Arias-Montaño 0000-0002-0791-8397; Angélica Rueda 0000-0001-8749-8494.

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Pamela Gaitán-González, Angélica Rueda, Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de México 07360, Mexico

Rommel Sánchez-Hernández, José-Antonio Arias-Montaño, Department of Physiology, Biophysics and Neurosciences, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de México 07360, Mexico

Corresponding author: Angélica Rueda, PhD, Professor, Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Av. IPN 2508, San Pedro Zacatenco, Ciudad de México 07360, Mexico. arueda@cinvestav.mx

Abstract

Metabolic syndrome is a pre-diabetic state characterized by several biochemical and physiological alterations, including insulin resistance, visceral fat accumulation, and dyslipidemias, which increase the risk for developing cardiovascular disease. Metabolic syndrome is associated with augmented sympathetic tone, which could account for the etiology of pre-diabetic cardiomyopathy. This review summarizes the current knowledge of the pathophysiological consequences of enhanced and sustained β-adrenergic response in pre-diabetes, focusing on cardiac dysfunction reported in diet-induced experimental models of pre-diabetic cardiomyopathy. The research reviewed indicates that both protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II play important roles in functional responses mediated by β_1-adrenoceptors; therefore, alterations in the expression or function of these kinases can be deleterious. This review also outlines recent information on the role of protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II in abnormal Ca^{2+} handling by cardiomyocytes from diet-induced models of pre-diabetic cardiomyopathy.

Key Words: Ca^{2+}/calmodulin-dependent protein kinase II; Protein kinase A; Metabolic syndrome; Pre-diabetes; Pre-diabetic cardiomyopathy; β-Adrenoceptors

Core Tip: Metabolic syndrome affects heart function leading to pre-diabetic cardiomyopathy. In an attempt to overcome contractility dysfunction, the activity of the

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sympathetic nervous system increases, but chronic stimulation of β-adrenoceptors leads to alterations in both protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II activity, the main effectors of the β-adrenergic response. This work recapitulates current evidence about the participation of protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II in experimental pre-diabetic cardiomyopathy, emphasizing the prevailing role of CaMKII in the development of cardiomyocyte Ca^{2+} mishandling and myocardial dysfunction associated with pre-diabetes.

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INTRODUCTION

Pre-diabetes, a high-risk state for the development of type 2 diabetes mellitus (DM2), is a condition where glycemia is higher than normal but not yet high enough for DM2 diagnosis[1,2]. According to the American Diabetes Association this condition is identified by laboratory tests, including fasting blood glucose (FBG) values 100-125 mg/dL, glycated hemoglobin in the range of 5.7%-6.4% or 2 h blood glucose values 140-199 mg/dL (75-g oral glucose tolerance test)[2-4].

Metabolic syndrome (MetS) is considered a pre-diabetic state and currently represents a serious public health problem because of its increasing worldwide prevalence. MetS comprises a cluster of biochemical and physiological alterations that become risk factors for cardiovascular disease (CVD)[3]. Key components of MetS are central obesity, elevated triglyceride levels, low high-density lipoprotein cholesterol levels, high blood pressure, and dysglycemia. Insulin resistance (IR) is the critical factor underlying MetS, although the pathogenesis remains unclear. Furthermore, an important feature of MetS patients is the prevalence of a hyperadrenergic state that could account for the development of cardiac disease[5,6].

For DM2 patients, the term diabetic cardiomyopathy refers to the presence of Ca^{2+} mishandling, cardiomyocyte hypertrophy, apoptosis, and fibrosis, together with abnormal myocardial performance in the absence of hypertension, coronary artery disease, or valvular heart disease[7,8]. Although the clinical entity of pre-diabetic cardiomyopathy still lacks a universally accepted definition, studies have linked pre-diabetes to CVD. Each MetS component represents a risk factor for CVD; in combination, these components increase the rate and severity of CVD as it relates to several conditions including microvascular dysfunction, coronary atherosclerosis and calcification, and cardiac dysfunction, which lead to myocardial infarction and heart failure (HF)[3]. In animal models of pre-diabetes, obesity, IR, and other components of MetS can lead to cardiac dysfunction associated with structural and functional abnormalities (Table 1), implying cardiomyopathy mechanisms different from those of DM2. Furthermore, observational studies and large sample meta-analyses show that pre-diabetes, defined as impaired glucose tolerance, impaired FBG, or raised glycated hemoglobin, was associated with increased risk of CVD[9,10] and HF[11]. Moreover, meta-analysis of longitudinal studies indicates that MetS is linked to increased risk of myocardial infarction, stroke, and CVD, with the risk estimate being higher than that corresponding to its individual components[12,13]. A disturbing finding is that young pre-diabetic patients with evident impaired FBG levels show increased prevalence of left ventricular hypertrophy, reflecting that heart damage is already present at an early phase of glucose metabolism alteration[14]. Patients with obesity, dyslipidemia, or IR (MetS components) are likely to develop similar metabolism-related cardiomyopathy even in the absence of diabetes[15] However, the mechanisms involved in the pathogenesis of what must be considered pre-diabetic cardiomyopathy remain poorly understood. For the purpose of this review, we will refer to IR-induced cardiomyopathy, obesity-related cardiomyopathy, or MetS-induced cardiomyopathy as 'pre-diabetic cardiomyopathy.'

Several reviews address the contribution of altered cardiac metabolism to dysfunction[7,15-18]; in this work we focus primarily on the possible link between pre-

Table 1 Characteristics of experimental models of pre-diabetic cardiomyopathy

Animal model	MetS parameters						Cardiovascular dysfunction	Ref.
	BW	BP	BG	IR	TG	HDL-C		
Dogs								
HFD dogs (80% of calories from fat, 5 wk)	↑	↑	↔	+	ND	ND	↑ Heart rate; ↓ Myocardial oxygen delivery and metabolism; ↓ Cardiac index after exercising. ↑ Aortic pressure	Setty *et al*[51] and Dincer *et al*[73]
Rats								
Sucrose-fed Wistar rats (68%, 7-10 wk)	↔	ND	↔	+	ND	ND	↓ FS; + Systolic dysfunction	Dutta *et al*[68]
Sucrose-fed Sprague-Dawley rats (68%, 7-10 wk)	↔	ND	↔	+	ND	ND	↔ Heart hypertrophy; ↓ FS; + Systolic dysfunction	Hintz *et al*[67], and Hintz and Ren[72]
Sucrose-fed Wistar rats (30%, 17-24 wk)	↔	↑	ND	+	↑	↓	↔/↑ Heart rate; ↓ Ventricular pressure; ↑ Arrhythmia incidence after reperfusion	López-Acosta *et al*[56], and Carvajal and Baños [60]
Sucrose-fed Sprague-Dawley rats (32%, 10 wk)	↑	ND	↑	+	↑	ND	↔ Heart hypertrophy; ↓ FS and EF; ↑ Septum dimension	Vasanji *et al*[52]
Sucrose-fed Wistar rats (30%, 24 wk)	↑	↑	↔	ND	↑	↔	↔ Heart hypertrophy; + Systolic dysfunction; ↓ Cardiac cell contraction	Barrera-Lechuga *et al* [53] and Fernández-Miranda *et al*[70]
Sucrose-fed Wistar rats (30%, 35 wk)	↑	↔	ND	ND	ND	ND	↔ Heart rate; ↔ Heart hypertrophy; ↓ FS	Paulino *et al*[65]
Sucrose-fed Wistar rats (30%, 16-18 wk)	↔	↔	↔	+	↑	ND	+ Systolic dysfunction	Balderas-Villalobos *et al* [69]
Sucrose-fed Wistar rats (20%, 8 wk)	↑	ND	↔	+	↔	↓	↓ Heart rate; ↑ SAN rate variability; ↑ SAN fat deposits	Albarado-Ibañez *et al* [54]
Sucrose-fed Wistar rats (32%, 16 wk)	↑	↑	↑	+	↑	ND	↑ Heart rate; ↑ Heart hypertrophy; ↓ Heart contractility; ↑ Cardiomyocyte lipid deposits; ↑ Aortic pressure	Okatan *et al*[27,28]
Fructose-fed Wistar rats (10%, 3 wk)	↔	↔	↔	+	↑	↓	↓ Heart rate; ↑ Heart hypertrophy; ↓ FS; ↓ Heart contractility; + Systolic dysfunction; + Diastolic dysfunction; + LV hypertrophy; ↑ Arrhythmia incidence	Sommese *et al*[55]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	↑	↔	↑	+	↔	↔	↔ Heart rate; ↔ Heart hypertrophy; ↔ FS; ↑ Lipid in the myocardium; + Diastolic dysfunction	Koncsos *et al*[62]
Sucrose-fed Wistar rats (30%, 4 mo)	↑	ND	↔	+	↑	↓	↑ Heart rate; ↔ Heart hypertrophy; + Diastolic dysfunction; ↑ Arrhythmia incidence	Romero-García *et al*[48] and Landa-Galvan *et al* [57]
Mice								
Fructose-fed C57bl/6 mice (10%, 3 wk)	↔	↔	↔	+	ND	ND	↓ FS; + LV hypertrophy; + Systolic dysfunction	Federico *et al*[71]
HFD C57bl/6 mice (60% of calories from fat, 8 wk)	↑	ND	↑	ND	↔	ND	↑ Heart rate; ↔ FS; ↑ Arrhythmia incidence	Sánchez *et al*[63]
HFD FVB-mice (45% of calories from fat, 5 mo)	↑	↔	ND	+	ND	ND	↔ Heart rate; ↑ Heart hypertrophy; ↓ FS; + Systolic dysfunction	Dong *et al*[66]

↔: No change; +: Presence; ↓: Decreased; ↑: Increased; BG: Blood glucose; BP: Blood pressure; BW: Body weight; EF: Ejection fraction; FS: Fractional shortening; HDL-C: High-density lipoprotein cholesterol; HFD: High-fat diet; IR: Insulin resistance; LV: Left ventricle; MetS: Metabolic syndrome; SAN: Sinus Atrial Node; STZ: Streptozotocin; TG: Triglycerides; ND: Not determined.

diabetic cardiomyopathy and alterations in the β-adrenergic system and two main downstream signaling effectors: cAMP-dependent protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

In cardiac cells, the expression and activity of key Ca^{2+} handling proteins involved in excitation-contraction coupling (ECC) are altered in IR and diabetic cardiomyopathy [8]. Under physiological conditions, cardiac ECC begins with Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels. A small influx of Ca^{2+} activates the intracellular Ca^{2+} channel/ryanodine receptor (RyR) through a mechanism known as Ca^{2+}-induced Ca^{2+} release, eliciting a transient Ca^{2+} increase in the cytoplasm of the cardiac cell that in turn activates the contractile machinery. Relaxation involves the clearance of intracellular Ca^{2+} by: (1) Re-uptake into the sarcoplasmic reticulum Ca^{2+} stores through the activity of the sarcoplasmic reticulum Ca^{2+} ATPase; and (2) Ca^{2+} extrusion by the Na^{+}/Ca^{2+} exchanger in the sarcolemma[19].

The β-adrenergic response is the main regulatory pathway of ECC, involving the activation of PKA and CaMKII. These kinases phosphorylate several Ca^{2+} handling proteins, including L-type voltage-dependent Ca^{2+} channels, RyRs, and phospholamban (PLN), thereby modifying their activity[20] (Figure 1). In this review, we summarize the recent evidence of alterations in the expression and/or activity of PKA and CaMKII in diet-induced animal models of pre-diabetic cardiomyopathy. This work also emphasizes the prevailing role of CaMKII in the development of myocardial dysfunction associated with pre-diabetes (Figure 1).

β-ADRENERGIC RECEPTOR SIGNALING IN THE HEART: PKA AND CAMKII ACTIVATION

The heart is innervated by parasympathetic and sympathetic fibers that regulate contractility rate and force. Sympathetic innervation of the atria and ventricles is provided by the stellate ganglion, whereas the vagus nerve provides parasympathetic fibers to the sinoatrial node, atrioventricular node, and atria[21].

Sympathetic fibers synthesize and release noradrenaline (NA), while chromaffin cells located in the medulla of adrenal glands synthesize and release adrenaline (A) into the bloodstream. Both catecholamines exert their functional effects through the activation of selective receptors, called adrenoceptors (ARs)[22]. ARs are divided into three families: α_1, α_2, and β. The α_1-AR family is composed of α_{1A}, α_{1B}, and α_{1D} receptors, the α_2-AR family by α_{2A}, α_{2B}, and α_{2C} subtypes, and the β-AR family comprises the β_1, β_2, and β_3 receptors. All three α_1-AR subtypes couple predominantly to $G\alpha_{q/11}$ proteins; their activation leads to phospholipase C stimulation, activation of protein kinase C, and inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores[23]. α_2-ARs couple to $G\alpha_{i/o}$ proteins, reducing cAMP formation, and inhibiting N- and P-type voltage-activated Ca^{2+} channels[24]. β-ARs mainly couple to $G\alpha_s$ proteins, eliciting adenylyl cyclase (AC) activation and cAMP formation[22] (see below), although stimulation of β_2- and β_3-ARs also activates $G\alpha_{i/o}$ proteins[25,26].

The regulation of cardiac function by the sympathetic nervous system *via* β-ARs is of particular interest because dysregulation of this system has been reported in HF and metabolic disorders such as DM2 and MetS[27-29].

Radioligand binding assays with human heart preparations indicate that cardiac tissues express mainly β_1- and β_2-ARs, which represent 90% of all ARs and are expressed at an 8:2 ratio in both atria and ventricles[30]. There is also evidence for the expression of β_3-ARs in cardiomyocytes[26]; however, the β_1 and α_{1B} subtypes are the main ARs expressed in isolated mouse ventricular cardiomyocytes, with β_2- and β_3-ARs expressed by only 5% of cardiomyocytes but with high expression by endothelial cells[31]. These data support the notion that β-adrenergic responses in cardiomyocytes are primarily mediated by β_1-ARs.

Furthermore, β_1-ARs are located on the surface of all cardiomyocytes, whereas β_2-ARs are expressed exclusively at T-tubules. However, in HF, β-AR expression is redistributed so that β_2-ARs co-localize with β_1-ARs[32], suggesting that β-ARs participate in the cardiac remodeling that underlies the pathogenesis of cardiac diseases.

β-ARs are activated by both NA and A, but the subtypes show different affinity for the endogenous agonists, with rank order of potency: β_1-ARs, NA > A; β_2-ARs, A > NA; and β_3-ARs, NA ≈ A[22]. As mentioned above, agonist-bound β-ARs stimulate AC activity *via* $G\alpha_s$ proteins. There are nine isoforms of membrane-integral ACs[33]; cardiac tissues primarily express the AC5 and AC6 isoforms[34]. ACs catalyze the synthesis of cAMP from ATP; cAMP directly activates PKA and the exchange protein directly activated by cAMP (Epac). These proteins participate in the activation of CaMKII *via* PKA-mediated increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the Epac/phosphoinositide 3-kinase/Akt/n-nitric oxide synthase pathway,

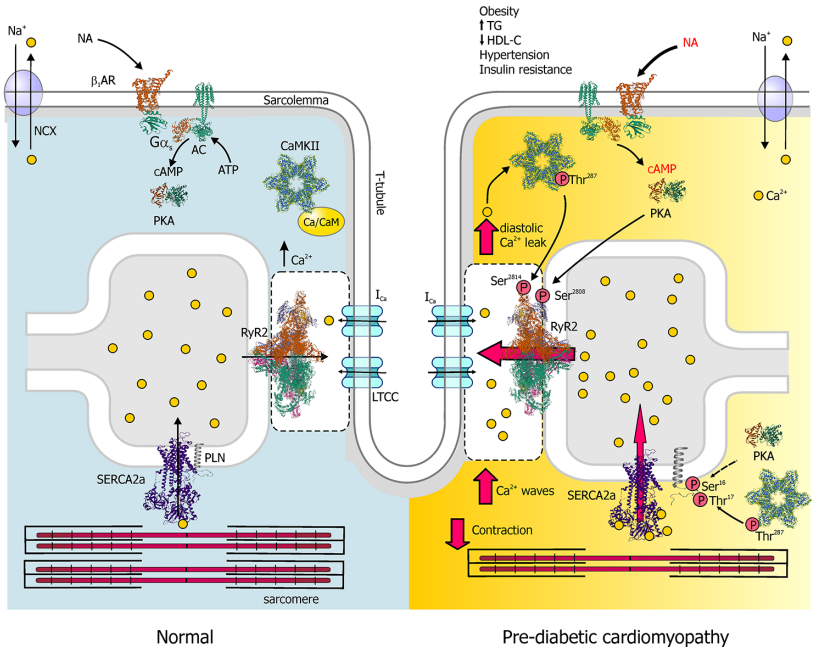

Figure 1 β-Adrenergic stimulation in the normal heart and pre-diabetic cardiomyopathy. Left panel: β-adrenergic stimulation in the normal heart. In physiological excitation-contraction coupling, membrane depolarization activates L-type voltage-dependent Ca^{2+} channels, inducing a small Ca^{2+} influx (*ICa*) that triggers the activation of cardiac ryanodine receptors (RyR2, PDB accession code: 6WOV). This triggers the release of sufficient Ca^{2+} from the lumen of the sarcoplasmic reticulum to the cytoplasm to elicit contraction. During relaxation, Ca^{2+} is primarily removed from the cytoplasm by the sarcoplasmic reticulum Ca^{2+} ATPase (PDB accession code: 6HXB), which resequesters Ca^{2+} into the sarcoplasmic reticulum lumen. Ca^{2+} is also extruded by the Na^+/Ca^{2+} exchanger (PDB accession code: 3US9), while a small amount of Ca^{2+} is taken up by the mitochondrial calcium uniporter (PDB accession code: 6WDN). Noradrenaline activates β_1-adrenoceptors (β_1-ARs, PDB accession code: 6H70) located at the sarcolemma of cardiomyocytes; agonist-bound β_1-ARs stimulate Ga_s proteins and therefore one or more isoforms of adenylyl cyclase (PDB accession code: 6R3Q), leading to cAMP formation and the activation of the cAMP-dependent protein kinase (PDB accession code: 3FHI). Protein kinase A phosphorylates several Ca^{2+} handling proteins, including RyR2 at Ser^{2808} and phospholamban (PDB accession code: 2LPF) at Ser^{16}; the latter increases sarcoplasmic reticulum Ca^{2+} ATPase pump activity. Ca^{2+} binds to calmodulin, and the complex Ca/calmodulin binds to and activates Ca^{2+}/calmodulin-dependent protein kinase II (PDB accession code: 3SOA), which phosphorylates RyR at Ser^{2814} and phospholamban at Thr^{17}. The exchange protein directly activated by cAMP (Epac) is also involved in Ca^{2+}/calmodulin-dependent protein kinase II activation; however, its role in pre-diabetic cardiomyopathy has not yet been addressed; thus, it is not depicted in the figure. Right panel: β-adrenergic stimulation in pre-diabetic cardiomyopathy. In the presence of obesity, increased triglyceride levels, decreased high-density lipoprotein cholesterol, hypertension, and/or insulin resistance (all Metabolic Syndrome components), and abnormal β_1-AR activation (associated with either chronic sympathetic tone or changes in β-AR expression) dysregulates excitation-contraction coupling in cardiac cells. Pre-diabetic cardiomyopathy is characterized by abnormal diastolic Ca^{2+} leak (diastolic dysfunction) due to augmented RyR2 phosphorylation at Ser^{2808} and Ser^{2814} in the absence of adrenergic stimulation, generating spontaneous Ca^{2+} waves that may induce pro-arrhythmogenic events through altered Na^+/Ca^{2+} exchanger activity. In addition, phosphorylated phospholamban (at Ser^{16} and Thr^{17}) detaches from sarcoplasmic reticulum Ca^{2+} ATPase 2a, augmenting its activity; finally, Ca^{2+} transient amplitude decreases and leads to impaired cell contraction. NA: Noradrenaline; AR: Adrenoceptors; NCX: Na^+/Ca^{2+} exchanger; AC: Adenylyl cyclase; PKA: Protein kinase A; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; CaM: Calmodulin; RyR: Ryanodine receptor; LTCC: L-type voltage-dependent Ca^{2+} channels; PLN: Phospholamban; HDL-C: High-density lipoprotein cholesterol; TG: Triglycerides; SERCA: Sarcoplasmic reticulum Ca^{2+} ATPase.

respectively[35]. In turn, both PKA and CaMKII phosphorylate several proteins involved in cardiac ECC, such as L-type voltage-dependent Ca^{2+} channels, RyR2, and PLN, leading to increased heart rate and contractile force[19,36].

PKA is a serine/threonine kinase comprising two regulatory (R) and two catalytic (C) subunits. There are four isoforms of the catalytic subunit (Cα, Cβ, CΥ, Cχ) and four isoforms of the regulatory subunit (RIα, RIIα, RIβ, RIIβ[37]). The PKA complex is formed by two catalytic subunits and two regulatory subunits; the complexes are named according to the number of the regulatory subunit (*i.e.* PKA-I and PKA-II). The regulatory subunits contain two cAMP binding sites and a pseudo-substrate domain that binds to the active site of the catalytic subunit in the absence of cAMP. The binding of two cAMP molecules to each regulatory subunit induces a conformational change that promotes the dissociation of the catalytic subunits from the regulatory subunits[38].

Cardiomyocytes express the four isoforms of the PKA regulatory subunits, with the α-isoforms being more abundant than the β-isoforms[39,40]. By using fluorescence resonance energy transfer-based cAMP reporters, Di Benedetto *et al*[41] showed that PKA-I and PKA-II are compartmentalized in cardiomyocytes through their binding to specific A-kinase anchoring proteins. PKA-I is expressed in a tightly striated manner that overlies the sarcomere Z and M lines, whereas PKA-II is strongly expressed in M lines and only slightly in Z lines. β-AR activation with the non-selective agonist

isoproterenol increases cAMP levels primarily in the PKA-II domain, leading to phosphorylation of the regulatory proteins troponin I and PLN as well as RyR2 at Serine 2808 (Ser^{2808}), among other residues. The effect of the latter results in increased RyR2 open probability, although the exact impact on channel function is not clear[41-43]. Together, these findings suggest that PKA-II, rather than PKA-I, underlies the functional responses mediated by β_1-ARs.

CaMKII also phosphorylates proteins involved in cardiac ECC[36]. Four CaMKII isoforms (α, β, ϒ, δ) have been reported; CaMKII-δ is the dominant isoform in cardiomyocytes[44]. CaMKII is a multimer complex of 12 monomers assembled in two hexameric rings; each monomer consists of an N-terminal domain, an autoinhibitory regulatory region, and a C-terminal domain. Transient increases in $[Ca^{2+}]_i$ are sensed by calmodulin, leading to the assembly of a Ca^{2+}/calmodulin complex, which binds to the CaMKII autoinhibitory regulatory domain and induces conformational changes that result in kinase activation and under some pathological conditions in CaMKII autophosphorylation at Thr^{287}[45]. In addition, several other post-translational modifications promote autonomous CaMKII activity, such as oxidation at $Met^{281/282}$, O-GlcNAcylation at Ser^{280}, and S-nitrosylation at Cys^{290}[35].

Emerging evidence supports a relevant role for Epac as a mediator of cAMP signaling in the heart. There are two Epac isoforms in mammals, Epac1 and Epac2; both contain an N-terminal regulatory domain and a C-terminal catalytic region. Upon cAMP binding, Epac proteins activate the Ras superfamily small GTPases Rap1 and Rap2[46]. CaMKII can also be activated by Epac2; in rat myocytes, the activation of β_1-ARs, but not β_2-ARs, lead to Epac2-dependent CaMKII-δ stimulation, which results in RyR2 phosphorylation at Ser^{2814}. This effect is abolished in CamKII-δ-KO mice, supporting a key role for this CaMKII isoform in cardiac responses mediated by β_1-ARs[47].

The research reviewed above suggests that both PKA and CaMKII-δ play important roles in β_1-AR-mediated responses and that alterations in the expression or function of these kinases can therefore be deleterious. Moreover, enhanced and sustained β-adrenergic stimulation contributes to the development of such pathological conditions as HF[29]; these alterations may also extend to diabetic and pre-diabetic cardiomyopathy[28,35,48]. A recent study showed that incubation of isolated mouse cardiomyocytes in high extracellular glucose (30 mmol/L) to mimic acute hyperglycemia leads to O-GlcNAcylation at CaMKII Ser^{280} and enhanced kinase activity, resulting in RyR2 phosphorylation and pro-arrhythmogenic activity[45]. Despite the availability of several MetS experimental models, pre-diabetic cardiomyopathy has been less studied (see below); the role of PKA and CaMKII in this pathology remains to be elucidated.

ANIMAL MODELS OF PRE-DIABETIC CARDIOMYOPATHY

Very few articles have considered MetS-associated cardiac alterations as pre-diabetic cardiomyopathy[49], most likely due to the lack of an accepted definition. Based on the graded effect of impaired glucose metabolism on diastolic function, it has been proposed that a morphological intermediate state between normal and diabetic states underlies pre-diabetic heart dysfunction[50]. One feature that perhaps differentiates pre-diabetic from diabetic cardiomyopathy is the absence of overt structural changes in the heart in the former, although this interpretation is under discussion[14].

Due to the multifactorial nature of cardiometabolic disease associated with obesity, IR, high blood pressure, high glycemic levels, and hypertriglyceridemia, the selection of an appropriate experimental model bearing the features of diet-induced pre-diabetic cardiomyopathy in humans has proven difficult. Most studies addressing diet-induced cardiometabolic alterations have been performed with laboratory animals under either carbohydrate- or fat and carbohydrate-enriched diets to emulate the Western diet, characterized by the ingestion of refined sugar and high caloric food. However, not all models — indeed, only eight[27,51-57] of those considered in this work — fulfill the requirement of at least three of the aforementioned criteria to be considered experimental models of MetS (Table 1).

Rats and mice are the most used animals for MetS models based on dietary manipulation; there are comprehensive reviews on this topic[58,59]. In this review, we focus on animal models with diet-induced pre-diabetic cardiomyopathy. Because the incidence of MetS in human populations is increasing, the establishment of MetS animal models is key to understanding the molecular mechanisms that are altered during the onset of myocardial disease. Although these diet-based experimental

models represent a critical milestone for pre-diabetic cardiomyopathy research, their utility is hampered by discrepancies in biochemical and corporal parameters, along with dissimilar outcomes that might be associated with the type and length of the diet. For instance, for 16 diet-induced models of pre-diabetic cardiomyopathy considered in this review, 10 showed a significant increase in body weight (Table 1), while only four models developed high blood pressure[28,51,53,60]. Also, in good agreement with a seminal report by Reaven[61], a hallmark feature of pre-diabetic cardiomyopathy models is the presence of IR. FBG levels were evaluated in 13 models, but only 4 reported altered values[28,52,62,63]. For dyslipidemia, high blood triglyceride levels were reported for seven models, and only four showed decreased blood high-density lipoprotein cholesterol levels[54,55,57,64] (Table 1).

Importantly, despite the discrepancies in metabolic alterations all these animal models developed pre-diabetic cardiomyopathy, characterized by several cardiac alterations. For instance, increased heart rate was reported in five models[27,48,51,56,63]; however, other studies in which this parameter was evaluated did not report changes[60,62,65,66]. Systolic dysfunction has also been observed, including decreased heart contractility, ventricular pressure, and intracellular Ca^{2+} transient amplitude[27,55,60,67-71], along with reduced fractional shortening[55,65-67,70-72] (Table 1). Diastolic dysfunction is also manifested by increased diastolic Ca^{2+} leak in the form of Ca^{2+} waves, without altering cytoplasmic Ca^{2+} levels[48,55,62]. To compensate for compromised cardiac output, the heart grows; however, few studies have documented either heart hypertrophy[27,55,66] or left ventricle hypertrophy[55,71]. Interestingly, several pre-diabetic cardiomyopathy models develop increased aortic pressure[27,28,51,73] and high arrhythmia incidence under basal or stressful conditions[48,55,56,63] (Table 1). Of note, rats and mice are the most common animal models for inducing pre-diabetic cardiomyopathy, although pigs and dogs have also been employed because of their greater degree of similarity to human cardiac physiology, including ionic currents that contribute to the cardiac action potential[74], Ca^{2+} removal mechanisms, and ECC regulatory mechanisms[19]. It is thus essential to select the appropriate experimental model considering the objectives of the study to be performed.

β-AR/AC/cAMP/PKA AXIS IN PRE-DIABETIC CARDIOMYOPATHY

As mentioned above, β-AR activation modulates ECC; cardiac dysfunction can therefore develop following alterations in the signaling pathways triggered by β-AR activation. Several studies have focused on PKA and CaMKII function (Table 2), which are effectors of β-adrenergic responses and the main topic of this review. However, the mechanisms by which the β-adrenergic pathway is disturbed in MetS are not yet clear; thus, it is important to understand how the βAR/AC/cAMP/PKA axis is affected, and how these changes originate or exacerbate cardiac dysfunction. In this section, we will describe the alterations in this signaling pathway reported in MetS and compare them with previous results found in DM.

Pre-diabetic cardiomyopathy can involve over-activation of the β-AR response. Indeed, patients with MetS show increased sympathetic activation, as measured by microneurography[75]; further, a cross-sectional and longitudinal study reported that MetS is associated with increased resting heart rate[76]. Both studies suggest over-activation of sympathetic activity by MetS, and we recently reported increased basal heart rate in the rat sucrose-induced MetS model[48]. Moreover, following the administration of an arrhythmogenic cocktail (caffeine 80 mg/kg and epinephrine 2 mg/kg; intravenously), 80% of the animals developed ventricular fibrillation, which suggests altered β-AR-mediated responses.

The reported alterations could also be related to changes in β-AR expression. For example, two studies in streptozotocin-induced diabetic rats (an experimental model of type 1 DM) reported a reduction in β_1-*AR* mRNA levels, but increased levels of both β_2- and β_3-*AR* mRNA. Conversely, the protein content of β_1- and β_2-ARs was reduced but that of β_3-ARs was increased[27,77], suggesting β-AR expression remodeling in the diabetic heart. However, β_1- and β_2-AR protein levels were not affected in a rat model of obesity with IR and hypertriglyceridemia[78] or a diet-induced MetS mouse model [79]. Of note, the study by Okatan *et al*[27] also evaluated β-AR expression in rats with MetS. The authors reported unaltered mRNA levels but diminished protein levels of β_1- and β_2-ARs, accompanied by normal cardiac function (as evaluated by left ventricle developed pressure following stimulation with NA)[27]. These findings suggest that an increased β-AR-mediated response compensates for the reduction in β_1- and β_2-AR

Table 2 Alterations in protein kinase A in experimental models of pre-diabetes induced by diet

Experimental model	Kinase modification	Functional effects	Ref.
HFD dogs (80% of calories from fat, 5 wk)	ND	↑ RyR2- Ser^{2809} phosphorylation	Dincer *et al*[73]
Sucrose-fed Sprague-Dawley rats (32%, 10 wk)	↑ PKA activity (kemptide phosphorylation)	↓ PLN-Ser^{16} phosphorylation	Vasanji *et al*[52]
Sucrose-fed Wistar rats (30%, 35 wk)	↔ expression and activity	↔ PLN-Ser^{16} phosphorylation; ↓ RyR2- Ser^{2808} phosphorylation	Paulino *et al*[65]
Sucrose-fed Wistar rats (32%, 16 wk)	↑ PKA activity (Thr^{198} phosphorylation)	↑ RyR2- Ser^{2808} phosphorylation; ↓ PLN-Ser^{16} phosphorylation	Okatan *et al*[28]
Fructose-fed Wistar rats(10%, 3 wk)	ND	↔ RyR2- Ser^{2808} phosphorylation	Sommese *et al*[55]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	ND	↔ PLN-Ser^{16} phosphorylation	Koncsos *et al*[62]
HFD C57bl/6 mice (60% of calories from fat, 8 wk)	ND	↔ RyR2- Ser^{2808} phosphorylation; ↔ PLN-Ser^{16} phosphorylation	Sánchez *et al*[63]
Sucrose-fed Wistar rats (30%, 24 wk)	ND	↔ RyR2- Ser^{2808} phosphorylation; ↔ PLN-Ser^{16} phosphorylation	Fernández-Miranda *et al*[70]
Sucrose-fed Wistar rats (30%, 4 mo)	ND	↔ RyR2- Ser^{2808} phosphorylation; ↔ PLN-Ser^{16} phosphorylation	Romero-García *et al*[48]
HFD C57bl/6N mice (45% of total calories from fat, 8 wk)	ND	↔ PLN-Ser^{16} phosphorylation	Llano-Diez *et al*[79]

↔: No change; ↓: Decreased; ↑: Increased; ND: Not determined; HFD: High-fat diet; PKA: Protein kinase A; PLN: Phospholamban; RyR2: Ryanodine receptor type 2; STZ: Streptozotocin.

expression in MetS. Nevertheless, further research is required to fully elucidate the link between MetS, β_1-AR expression, signaling alterations, and cardiac dysfunction.

β-AR stimulation results in AC activation *via* $G\alpha_s$ proteins. However, we found no studies that evaluated $G\alpha_s$ expression or activity in MetS experimental models, although decreased $G\alpha_s$ protein expression was reported for diabetic Yucatan minipigs [80]. Furthermore, AC activity was normal in ventricular preparations from obese rabbits[81], which would suggest that cAMP intracellular concentration is unchanged; however, AC activity has not been studied in MetS models.

PKA is activated by cAMP and contributes to enhanced heart rate and contractility by phosphorylating several proteins, including RyR2 and PLN. In streptozotocin-induced diabetic mice, both PKA activity and cytosolic PKA catalytic subunit content were reduced[82]. Further, in rat isolated cardiomyocytes, incubation in medium supplemented with high glucose (25.5 mmol/L) reduced PKA activity[83]. Finally, PKA activity diminished along with a reduction in the positive inotropic response induced by isoproterenol in obese diabetic Zucker rats[84]. Together, these studies indicate that hyperglycemic conditions affect PKA function.

Three studies have evaluated PKA activity in pre-diabetic models: Okatan *et al*[28] and Vasanji *et al*[52] reported increased kinase activity, but Paulino *et al*[65] did not detect significant changes (Table 2). PKA activity has also been studied indirectly by determining the phosphorylation levels of PLN (Ser^{16}) or RyR2 (Ser^{2808} in rats; Ser^{2809} in dogs), with contradictory results (Table 2). Two studies reported increased RyR2 phosphorylation[28,73], one a decrease[65], and four lack of effect[48,55,63,70], while reduced PLN-Ser^{16} phosphorylation was found in two studies[28,52], and six reported no change[48,62,63,65,70,79].

Furthermore, upregulated PKA expression was reported for a genetic MetS model, a double knock-out of LDL-receptor (LDLR-/-) and leptin-deficient (*ob/ob*) murine model, likely indicating that the genetic background contributes to the phenotype of the pathology[85]. Thus, the observed variations in PKA function could be due to the different conditions to which the animals were exposed, for example, diet composition and length (Table 2).

In summary, several studies found alterations in heart function or cardiomyocyte contraction in diet-induced models of pre-diabetes, which could be associated with altered PKA activity (Table 2). Because only three studies directly evaluated kinase activity[28,52,65] and reported contradictory results, more work is needed to determine the precise role of PKA in pre-diabetic cardiomyopathy. Together, the information reviewed suggests modification of the β-AR/AC/cAMP/PKA signaling

pathway upstream of PKA or disruption of other effectors of the β-adrenergic response, such as CaMKII, which are not yet broadly studied in MetS. We found no data on PKA alterations in diabetic or pre-diabetic patients; clearly studies addressing this issue would provide valuable information on the pathophysiology of MetS- and diabetes-induced cardiomyopathy.

CAMKII AS A NOVEL TARGET IN PRE-DIABETIC CARDIOMYOPATHY

CaMKII has been proposed as a key contributor to the deleterious effects of chronic β-AR activation in diabetic cardiomyopathy, primarily by exacerbating RyR2-mediated diastolic Ca^{2+} leak[86,87]. Studies in experimental models of IR and fructose fed-induced pre-diabetic cardiomyopathy have unveiled the role of hyperglycemia and reactive oxygen species in inducing abnormal CaMKII phosphorylation at Thr^{287} and activation, altering cardiomyocyte intracellular Ca^{2+} handling and promoting cardiac arrhythmic events[55,71,88]. Hyperglycemia leads to CaMKII glycosylation, increasing RyR2-mediated Ca^{2+} leak and reducing sarcoplasmic reticulum Ca^{2+} load in cardiac cells[88]. However, in pre-diabetic cardiomyopathy hyperglycemia is not overt[48]; thus, abnormal CaMKII activation relies on additional mechanisms[48,55]. The length of CaMKII activation relies on the frequency of Ca^{2+} release events, and extended CaMKII activation is also related to autophosphorylation at Thr^{287}, which prevents CaMKII auto-inhibition[87]. In animal models of pre-diabetes, cardiac CaMKII remains active even when the $[Ca^{2+}]_i$ declines, constituting a mechanism for anomalous CaMKII activation (Table 3)[48,55,87]. CaMKII phosphorylates RyR2 at Ser^{2814}; CaMKII abnormal activation can therefore induce higher activity of RyRs even at diastolic Ca^{2+} levels, leading to increased spontaneous Ca^{2+} wave frequency and propensity to spontaneous cardiomyocyte contraction and arrhythmias[48,55]. Of note, CaMKII activity was determined only in one study[52] (Table 3); therefore, further studies are needed.

In spontaneously hypertensive rats, which could be considered a genetic model of MetS, the knock-out of Camk2n1 (*SHR-Camk21*$^{-/-}$), a peptide that regulates the association of Ca^{2+}/calmodulin with CaMKII, reduced kinase activity in the heart, thereby improving cardiac function[89]. Interestingly, the effect of deleting CaMKII- in sucrose-induced cardiac dysfunction has not yet been evaluated.

In heart disease, CaMKII has been implicated in ECC disorders that lead to cardiac dysfunction[90]; in particular, CaMKII overactivation is associated with the appearance of arrhythmias linked to abnormal Ca^{2+} handling[91-93].

As mentioned above, phosphorylation at Ser^{2814} by abnormal CaMKII activation induces RyR2 hyperactivity. Thus, preventing RyR phosphorylation by a point mutation (Ser2814Ala) that inactivates the phosphorylation site of CaMKII circumvents the development of HF induced by transverse aortic constriction in mice [94]. In contrast, a mutation that mimics RyR2 constitutive activation by CaMKII exacerbates arrhythmogenesis and sudden cardiac death in mice with HF[95]. Moreover, in mice with HF, knock-out of the CaMKII ϒ/ isoforms protects against cardiac dysfunction and fibrosis induced by pressure overload and β-adrenergic stimulation[96,97]. Of note, in two diet-induced pre-diabetic cardiomyopathy models, pharmacological inhibition of CaMKII prevents Ca^{2+} mishandling and RyR dysregulation[48,55].

Notably, post-translational modifications (specifically, oxidation, O-glycosylation, and phosphorylation) of CaMKII are increased in heart samples of diabetic patients[88,98,99], suggesting altered kinase activity. As for PKA, research on the possible role of CaMKII alterations in diabetic or pre-diabetic patients is required to increase our understanding of the pathophysiology of MetS- and diabetes-induced cardiomyopathy.

CONCLUSION

MetS is a serious public health problem with increased risk for CVD and DM2, leading to cardiac dysfunction in the form of pre-diabetic cardiomyopathy. This, in turn, stimulates the β-adrenergic response with inotropic and chronotropic positive effects that initially compensate the deficient heart contraction but that eventually become deleterious in chronic disease.

Table 3 Alterations in Ca^{2+}/calmodulin-dependent protein kinase II in experimental models of pre-diabetes induced by diet

Experimental model	CaMKII alterations	Functional effects	Ref.
Sucrose-fed Sprague-Dawley rats (32% 10 wk)	↑ CaMKII activity (autocamtide phosphorylation)	↓ PLN-Thr^{17} phosphorylation	Vasanji *et al*[52]
Fructose-fed Wistar rats (10%, 3 wk)	↑ CaMKII oxidation; ↔ CaMKII expression	↑ RyR2-Ser^{2814} phosphorylation	Sommese *et al*[55]
Sucrose-fed Wistar rats (30%, 4 mo)	↑ CaMKII-Thr^{287} phosphorylation; ↔ CaMKII expression	↑ RyR2-Ser^{2814} phosphorylation	Romero-García *et al*[48]
HFD Long-Evans rats treated with STZ (40% lard, 21 wk)	↔ CaMKII-Thr^{287} phosphorylation; ↔ CaMKII expression	↔ PLN-Thr^{17} phosphorylation	Koncsos *et al*[62]

↔: No change; ↓: Decreased; ↑: Increased; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; HFD: High-fat diet; PLN: Phospholamban; RyR2: Ryanodine receptor type 2; STZ: Streptozotocin.

There is evidence supporting the hypothesis that MetS alters β-adrenergic signaling, but it is still not clear how β-adrenergic signaling is affected in diet-induced MetS models. β_1-ARs are the more abundant isoform in cardiomyocytes and are the primary mediators of the β-adrenergic response under physiological conditions. However, the link between MetS, β_1-AR expression and signaling alterations and cardiac dysfunction remains to be fully established.

β-AR stimulation leads to PKA and CaMKII activation, and MetS could involve kinase overactivation. For PKA, the available data indicate overactivation, no change, or reduced activity; further research is clearly needed. For CaMKII, the evidence suggests a critical role in the development of pre-diabetic cardiomyopathy; understanding the mechanisms that dysregulate CaMKII activity in MetS would therefore contribute importantly to elucidating the molecular basis of cardiac dys-function.

Importantly, the majority of the information reported in this review was generated with small rodent models; further studies are required in animal models that more closely approximate human cardiac physiology.

Future perspectives

Several issues remain to be addressed in investigating the possible effect of MetS on β-adrenergic signaling pathways in cardiomyocytes and actions on PKA and CaMKII activity. For instance, the role of Epac2, which is also activated by β_1-AR stimulation, has not been elucidated in pre-diabetic cardiomyopathy. Furthermore, it is not well established whether MetS modifies β-AR expression by cardiomyocytes or what role receptor desensitization might play in the hyper-adrenergic state induced by the syndrome. An additional relevant question is whether MetS induces post-translational modifications in CaMKII that result in an altered activity. Additional knowledge would allow for laying the foundation for the rational design of targeted therapies to prevent or treat the development of pre-diabetic cardiomyopathy.

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MINIREVIEWS

Glycemic targets in critically ill adults: A mini-review

Kay Choong See

ORCID number: Kay Choong See 0000-0003-2528-7282.

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Kay Choong See, Division of Respiratory and Critical Care Medicine, Department of Medicine, National University Hospital, Singapore 119228, Singapore

Corresponding author: Kay Choong See, FCCP, FRCP, MBBS, MRCP, Doctor, Division of Respiratory and Critical Care Medicine, Department of Medicine, National University Hospital, 1E Kent Ridge Road, NUHS Tower Block Level 10, Singapore 119228, Singapore. mdcskc@nus.edu.sg

Abstract

Illness-induced hyperglycemia impairs neutrophil function, increases pro-inflammatory cytokines, inhibits fibrinolysis, and promotes cellular damage. In turn, these mechanisms lead to pneumonia and surgical site infections, prolonged mechanical ventilation, prolonged hospitalization, and increased mortality. For optimal glucose control, blood glucose measurements need to be done accurately, frequently, and promptly. When choosing glycemic targets, one should keep the glycemic variability < 4 mmol/L and avoid targeting a lower limit of blood glucose < 4.4 mmol/L. The upper limit of blood glucose should be set according to casemix and the quality of glucose control. A lower glycemic target range (*i.e.*, blood glucose 4.5-7.8 mmol/L) would be favored for patients without diabetes mellitus, with traumatic brain injury, or who are at risk of surgical site infection. To avoid harm from hypoglycemia, strict adherence to glycemic control protocols and timely glucose measurements are required. In contrast, a higher glycemic target range (*i.e.*, blood glucose 7.8-10 mmol/L) would be favored as a default choice for medical-surgical patients and patients with diabetes mellitus. These targets may be modified if technical advances for blood glucose measurement and control can be achieved.

Key Words: Brain injuries; Traumatic; Critical care; Diabetes mellitus; Glycemic control; Insulin infusion systems; Sepsis

Core Tip: A lower glycemic target range (*i.e.*, blood glucose 4.5-7.8 mmol/L) would be favored for patients without diabetes mellitus, or with traumatic brain injury, or who are postoperative and at risk of surgical site infection. Requirements for targeting a lower range and avoiding hypoglycemia would be availability of intensive glucose monitoring and management, strict adherence to glycemic control protocols, and strict

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INTRODUCTION

Illness-induced hyperglycemia can be a double-edged sword. On the one hand, it may be an adaptive response to provide extra metabolic substrate to organs like the brain and to blood cells[1]. On the other hand, hyperglycemia impairs neutrophil function and innate immunity, increases pro-inflammatory cytokines and oxidative stress[2,3], inhibits fibrinolysis[4], and promotes cellular damage[1]. In addition, hyperglycemia in brain-injured patients can lead to microcirculatory damage, blood-brain barrier disruption, and cellular swelling[5]. These pathological derangements potentially lead to complications such as pneumonia and surgical site infections, prolonged mechanical ventilation, increased intensive care unit (ICU) and hospital lengths of stay, and increased mortality.

Unlike hyperglycemia, hypoglycemia is always harmful. For example, hypoglycemia was independently associated with respiratory complications and prolonged ICU and hospital lengths of stay after cardiac surgery[6]. These adverse events may be mediated by hypoglycemia-related neuronal damage and cardiac arrhythmia[7]. Apart from the clear need to avoid blood glucose extremes, there is also a need to avoid excessive blood glucose fluctuations[8], which can be measured in various ways (Table 1). The simplest measure of blood glucose fluctuation is glycemic variability, which is the difference between the maximum and minimum blood glucose measured over a defined time interval. At the cellular level, glycemic variability has been associated with oxidative stress, endothelial dysfunction, and apoptosis[7]. Clinically, glycemic variability has been linked to increased ICU and hospital mortality[9,10].

Blood glucose measurements need to be done accurately, frequently, and promptly [11]. Ideally, blood glucose measurements should be done continuously, though continuous glucose monitoring (CGM) for critically ill patients may not be accurate enough, with wide limits of agreement despite small mean bias[12]. CGM appears unreliable when using minimally-invasive subcutaneous devices that assay interstitial glucose measurements[13-15], and does not seem to improve glucose control[16]. Although invasive (intravascular) CGM devices may have an acceptable accuracy, some drawbacks include vascular and infectious complications (thrombosis, catheter occlusion, biofilm formation, or intravascular catheter-related infection)[17,18].

Accuracy and variation of glucose measurement methods influence the feasibility and adherence to glycemic targets[19]. In the real world, a variety of blood samples (arterial, venous, and capillary) are assayed intermittently, using both point-of-care and laboratory methods[20,21], and managed using various protocols. Nonetheless, despite such variation, clinical utility of current glucose measurement systems seems adequate, with little evidence of over or under-treatment[22]. Additionally, to achieve optimal clinical outcomes, blood glucose should be lowered if it were to rise too high, blood glucose should not be allowed to dip too low, and blood glucose variability should be constrained.

To determine clinically optimal glycemic targets for critically ill adult patients, the key questions would therefore be as follows: (1) What should the *hyperglycemic* threshold be; (2) What should the *hypoglycemic* threshold be; and (3) How far apart should these thresholds be? This review aims to integrate empirical evidence to answer these questions, and to suggest practical recommendations for choosing glycemic targets.

Table 1 Types of glycemic targets in intensive care unit

Glycemic target	Unit	Definition
Glucose	mmol/L	Concentration of glucose in blood or plasma. To convert to mg/dL, multiply by 18, *i.e.*, 1 mmol/L = 18 mg/dL
COV	%	Coefficient of variation, a measure of glucose variability. COV = standard deviation divided by mean glucose × 100%
GG	mmol/L	Glycemic gap. GG = blood glucose - [(1.59 × HbA1c) - 2.59], HbA1c being used to estimate average glucose concentration over the prior 3 mo
Glucose variability	mmol/L	Maximum – minimum glucose in a given time period
SHR	Nil	Stress hyperglycemia ratio. SHR = plasma glucose divided by [(1.59 × HbA1c)–2.59], HbA1c being used to estimate average glucose concentration over the prior 3 mo

HbA1c: Glycosylated hemoglobin.

EMPIRICAL EVIDENCE FOR GLYCEMIC THRESHOLDS IN ICU

Several trials are inconclusive with respect to intensive (lower) *vs* conventional (higher) glycemic targets, which may be due to insufficient separation of achieved glucose levels between the intervention and control groups[23-25]. Another reason could be that the impact of glucose control was modified by the main diagnosis (*i.e.*, casemix). In terms of the hyperglycemic threshold, the blood glucose level beyond which clinical complications occur seems to differ by casemix (Table 2). Patients without diabetes mellitus (DM)[26], patients with traumatic brain injury (TBI), and post-surgical patients at risk of wound infection experience adverse effects of hyperglycemia at a relatively low range, with the threshold set at 6.7-8.3 mmol/L[27-30].

The NICE-SUGAR trial showed that undifferentiated medical-surgical ICU patients had decreased 90-d mortality and incident hypoglycemia when the upper limit of blood glucose was set at 10 mmol/L rather than 6.1 mmol/L[31]. Patients who suffered non-TBI-specific injury[32] or who had post-cardiac arrest[33] also experienced better neurological recovery if blood glucose could be kept below 10 mmol/L.

Patients with prior DM were able to tolerate a higher mean blood glucose level (*i.e.*, blood glucose level > 10 mmol/L) without excess complications during critical illness, although these patients benefited from lowering blood glucose below 7.8 mmol/L after coronary artery bypass surgery[34]. Chronic hyperglycemia may have compensatory mechanisms in place that provide protection from acute hyperglycemia-related cellular damage[2]. The upper limit of safety in patients with DM appears to be a blood glucose level of 14 mmol/L[35].

In contrast to the risk of hyperglycemia differing by casemix, the risks of hypoglycemia appear to affect a broad range of patients similarly. Severe hypoglycemia (< 2.2 mmol/L), moderate hypoglycemia (< 3.3 mmol/L), and even mild hypoglycemia (<4 mmol/L) have been associated with ICU and hospital mortality[36-39]. Targeting lower blood glucose levels resulted in higher rates of severe hypoglycemia[40,41], and no clinical trial has targeted a lower limit of blood glucose < 4.4 mmol/L. The NICE-SUGAR trial demonstrated that the risk of hypoglycemia can be mitigated by avoiding targeting blood glucose below 6.1 mmol/L[31]. Nonetheless, if intensive glucose monitoring and management resources are available, and if glycemic control protocols and timely glucose measurements can be strictly adhered to, the Leuven studies demonstrated advantages of targeting blood glucose below 6.1 mmol/L, with surgical patients deriving clearer survival benefit and morbidity reduction compared to medical patients[23,42].

EMPIRICAL EVIDENCE FOR MINIMIZING GLYCEMIC VARIABILITY IN ICU

In a multicenter observational study, Egi *et al*[43] first showed that ICU non-survivors had a wider spread of glucose values compared to ICU survivors. Specifically, the standard deviation of blood glucose values was 2.3 mmol/L in non-survivors compared to 1.3 mmol/L in survivors. The association between spread of blood glucose with hospital mortality persisted after controlling for confounders (hospital

Table 2 Glycemic targets in intensive care unit by casemix and thresholds

Casemix	Blood sample	Method	Glycemic target	Evidence
Burns	Not stated	Not stated	Glucose > 7.8 mmol/L	Increased pneumonia, ventilator-associated pneumonia, and urinary tract infection; Obs[72]
Cardiac	Not stated	Not stated	Glucose 4.4-6.1 mmol/L	Decreased 30-d mortality compared to glucose 5-7.8 mmol/L; Obs[73]
DM	Not stated	Portable glucometer, blood gas analyzer	Glucose < 14 mmol/L	Decreased glycemic variability and incident hypoglycemia; before-and-after study[35]
DM	Arterial, venous	Blood gas analyzer	Glucose 10-14 mmol/L	Decreased incident hypoglycemia; before-and-after study[74]. No increased risk of hospital-acquired infectious, cardiovascular, renal or neurological complications; before-and-after study[75]
DM	Not stated	Portable glucometer	Glucose 5.6-7.8 mmol/L	Decreased complications (infection, cardiac events, respiratory failure, kidney failure) after coronary artery bypass graft surgery compared to glucose 7.8-10 mmol/L; RCT[34]
DM	Not stated	Portable glucometer	Glucose 5-7.8 mmol/L	Decreased 30-day mortality compared to glucose 4.4-6.1 mmol/L; Obs[76]
Medical	Capillary	Portable glucometer	Glucose > 7 mmol/L	Increased ICU mortality; Obs[77]
Medical-surgical	Arterial	Point-of-care or blood gas or laboratory analyzers	Glucose 8-10 mmol/L	Decreased 90-d mortality and incident severe hypoglycemia compared to glucose 4.5-6.0 mmol/L; RCT[31]
Medical-surgical	Not stated	Portable glucometer	Glucose 4.4-6.1 mmol/L	Decreased 30-d mortality compared to glucose 5-7.8 mmol/L in patients without DM; Obs[76]
Medical-surgical	Arterial	Point-of-care or blood gas or laboratory analyzers	Glucose 4.4-6.1 mmol/L	Increased incident severe hypoglycemia compared to more liberal control (95%CI of glucose -7.8-9.4) mmol/L; RCT[78]
Medical-surgical	Arterial, capillary	Glucometer	Glucose 10-11.1 mmol/L	Decreased incident severe hypoglycemia compared to glucose 4.4-6.1 mmol/L; RCT[46]
Medical-surgical	Arterial, capillary, venous	Glucometer or blood gas analyzer	Glucose 7.8-10 mmol/L	Decreased incident severe hypoglycemia compared to glucose 4.4-6.1 mmol/L; RCT[79]
Medical-surgical	Arterial	Portable glucometer	Glucose 7-9 mmol/L	Decreased ICU mortality compared to out-of-range glucose; Obs[80]
Medical-surgical	Arterial, capillary	Glucometer or blood gas analyzer	Glucose < 10 mmol/L	Decreased incident severe hypoglycemia compared to glucose 4.4-6.1 mmol/L; RCT[31,81]
Medical-surgical	Arterial	Glucometer	Glucose < 8 mmol/L	Decreased ICU mortality compared to higher glucose levels; Obs[82]
Medical-surgical	Arterial	Blood gas analyzer	Glucose > 8.3 mmol/L	Increased ICU mortality compared to glucose 6.1-8.3; Obs[83]
Medical-surgical	Arterial, capillary	Glucometer	Glucose < 8.2 mmol/L	Decreased ICU mortality compared to higher glucose levels; Obs[84]
Medical-surgical	Arterial, venous	Glucometer	Glucose 4.4-7.8 mmol/L	Decreased ICU and hospital mortality compared to glucose 7.8-10 mmol/L in patients without DM; Obs[26]
Medical-surgical	Not stated	Glucometer	Glucose 3.9-7.8 mmol/L	Time in range associated with decreased ICU mortality in patients without DM; Obs[85]; Time in range associated with decreased ICU mortality in patients receiving insulin; Obs[86]
Medical-surgical	Venous	Laboratory	Low SHR < 1	Decreased hospital mortality compared to SHR > 1 regardless of baseline HbA1c; Obs[87]
Post-CA	Capillary, venous	Not stated	Glucose 3.9-7.8 mmol/L	Higher survival, compared to higher glucose levels; Obs[88]
Post-CA	Not stated	Not stated	Glucose 4-10 mmol/L	Better neurological recovery, compared to higher glucose levels; Obs[33]
Surgical	Arterial	Blood gas analyzer	Glucose 4.4-6.1 mmol/L	Decreased hospital mortality, blood stream infections, acute renal failure, blood transfusion, critical-illness polyneuropathy, prolonged mechanical ventilation, compared to glucose 10-11.1 mmol/L; RCT[42]
Surgical	Not stated	Not stated	Glucose 4.4-6.1 mmol/L	Decreased post-operative renal failure and 30-d mortality compared to glucose > 8.3 mmol/L; Obs[89]

Surgical	Arterial, capillary, venous	Glucometer or blood gas analyzer	Glucose 4.4-7.8 mmol/L	Decreased hospital mortality compared to glucose >7.8 mmol/L; Obs[27]
Surgical	Not stated	Glucometer	Glucose 4-8 mmol/L	Decreased surgical site infection after coronary artery bypass graft surgery compared to glucose 4-10 mmol/L; before-and-after study[28]
Surgical	Arterial, venous	Continuous sensor, in a closed-loop system	Glucose 4.4-6.1 mmol/L	Decreased surgical site infection post- hepato-biliary-pancreatic surgery, compared to glucose 7.7-10.0 mmol/L; RCT[90]
Surgical	Arterial	Blood gas analyzer	Glucose 6.7-8.9 mmol/L	Decreased mortality compared to glucose 8.9-10 mmol/L; quasi-experimental (alternate allocation of participants)[91]
Surgical	Capillary	Glucometer	Glucose 6.1-8.3 mmol/L	Decreased surgical site infection and atrial fibrillation after coronary artery bypass graft surgery; before-and-after study[29]
TBI	Arterial	Blood gas analyzer	Glucose 3.5-6.5 mmol/L	Reduced intracranial hypertension and decreased rate of pneumonia, bacteremia and urinary tract infections during 2nd week, compared to glucose 5-8 mmol/L; Obs[5]
TBI	Not stated	Not stated	Glucose 4.4-6.7 mmol/L	Decreased risk of poor neurological outcomes but increased risk of hypoglycemia, and no mortality benefit, compared to higher glucose targets; systematic review of RCT[30]
TBI	Arterial	Point-of-care or blood gas or laboratory analyzers	Glucose 8-10 mmol/L	Decreased incident severe hypoglycemia, but no mortality benefit, compared to glucose 4.5-6.0 mmol/L; RCT[92]
TBI	Not stated	Not stated	Glucose < 11.1 mmol/L	Decreased hospital mortality compared to glucose > 11.1 mmol/L; Obs[93]
Trauma	Arterial, capillary, venous	Point-of-care or laboratory analyzers	Glucose < 7.8 mmol/L	Decreased ICU mortality compared to glucose > 7.8 mmol/L; Obs[94]
Trauma	Capillary	Not stated	Glucose < 10 mmol/L	Decreased hospital mortality compared to glucose > 10 mmol/L; Obs[32]

DM: Diabetes mellitus; HbA1c: Glycosylated hemoglobin; ICU: Intensive care unit; Obs: Observational study; RCT: Randomized controlled trial; SHR: Stress hyperglycemia ratio; TBI: Traumatic brain injury.

site, surgical patients, neurologic diseases, mechanical ventilation, acute physiological and chronic health evaluation II score, age, mean blood glucose level, maximum blood glucose level, and admission blood glucose level).

Subsequently, other observational studies have demonstrated that the difference between maximum and minimum blood glucose levels (*i.e.*, glucose variability) should not exceed 4-6 mmol/L, regardless of casemix[10,44,45] (Table 3). In other words, glycemic target ranges should ideally be < 4 mmol/L in width. Such a narrow range seems to be achievable, given that both single-center and multi-center randomized trials using a variety of protocols have successfully constrained glucose levels within standard deviations of < 2 mmol/L[23,31,42,46].

CHOOSING LOWER *VS* HIGHER GLYCEMIC TARGET RANGES

To minimize patient harm, empirical evidence suggests that when choosing glycemic targets, one should keep the glycemic variability < 4 mmol/L and avoid targeting a lower limit of blood glucose < 4.4 mmol/L. The upper limit of blood glucose should then be set according to casemix and the quality of glucose control.

A lower glycemic target range (*i.e.*, blood glucose 4.5-7.8 mmol/L) would be favored for patients without DM, with TBI, or who are postoperative and at risk of surgical site infection. Requirements for targeting a lower range and avoiding harm from hypoglycemia would be availability of intensive glucose monitoring and management, strict adherence to glycemic control protocols, and strict adherence to timely glucose measurements (Table 4).

In contrast, a higher glycemic target range (*i.e.*, blood glucose 7.8-10 mmol/L) would be favored as a default choice for medical-surgical patients and patients with DM. Additionally, a higher range would be favored if conditions to avoid hypoglycemia cannot be strictly met, *i.e.*, lack of intensive glucose monitoring and management, less than strict adherence to glycemic control protocols, and less than strict adherence to timely glucose measurements.

Table 3 Glycemic targets in intensive care unit by casemix and variability

Casemix	Blood sample	Method	Glycemic target	Evidence
Medical-surgical	Arterial, venous	Glucometer	Glucose variability (COV ≥ 20%)	Increased ICU and hospital mortality in patients without DM; Obs[26]
Medical-surgical	Arterial, capillary	Glucometer or blood gas analyzer	Glucose variability > 6 mmol/L	Increased ICU and hospital mortality; Obs[44]
Medical-surgical	Arterial	Glucometer or blood gas analyzer	Glucose variability > 4 mmol/L	Increased hospital mortality; Obs[10]
Post-CA	Arterial	Blood gas analyzer	Glucose variability < 5 mmol/L	Decreased hypoglycemia and mortality; Obs [45]
Post-CA	Not stated	Not stated	GG-min < 3.9 mmol/L	Better neurological recovery; Obs[95]

COV: Coefficient of variation; GG: Glycemic gap; GG-min: Minimum glycemic gap = minimum blood glucose - [(1.59 × HbA1c) - 2.59], HbA1c being used to estimate average glucose concentration over the prior 3 mo; ICU: Intensive care unit; Obs: Observational study.

Table 4 Choosing lower *vs* higher glycemic target ranges

Glycemic target range	Considerations favoring choice of glycemic target range
Lower glycemic target range (*i.e.*, glucose 4.5-7.8 mmol/L)	(1) Patients without DM; (2) Patients with TBI; (3) Post-surgical patients at risk of surgical site infections; (4) Availability of intensive glucose monitoring and management; (5) Strict adherence to glycemic control protocols; and (6) Strict adherence to timely glucose measurements
Higher glycemic target range (*i.e.*, glucose 7.8-10 mmol/L)	(1) Default choice for most patients; (2) Patients with DM; (3) Lack of intensive glucose monitoring and management; (4) Less than strict adherence to glycemic control protocols; and (5) Less than strict adherence to timely glucose measurements

DM: Diabetes mellitus; TBI: Traumatic brain injury.

This review's recommendations are in line with current guidelines (Table 5). For hospitalized patients in general, the American Diabetes Association recommends a glycemic target range of 7.8-10 mmol/L[47]. The same glycemic range is recommended for post-resuscitation care of cardiac arrest patients by the European Resuscitation Council[48]. For sepsis patients, the Surviving Sepsis Campaign recommends an upper blood glucose limit of 10 mmol/L[49]. Both the American Diabetes Association and Surviving Sepsis Campaign guidelines mention that lower targets may be appropriate for selected patients if they can be achieved without significant hypoglycemia[47,49].

Other guidelines have made less definite recommendations. For surgical patients, the World Health Organization recommends glucose control, though no target range was defined[50]. For patients with TBI, the Brain Trauma Foundation does not mention glycemic control[51]. The findings and recommendations from this review can therefore help fill any gaps in these latter guidelines.

FUTURE DIRECTIONS

To increase the safety of lower glycemic targets, technical advances for blood glucose measurement and control would help. Autocorrecting point-of-care glucose measurement devices can adjust for interfering substances (*e.g.*, ascorbic acid and non-glucose sugars) and abnormal hematocrit in critically ill patients[52], enabling these devices to become as accurate as central laboratory plasma glucose measurements. Monte Carlo simulation suggests that glycemic control in critically ill patients is optimal with a blood glucose measurement interval no longer than 1 h, with incremental benefit using shorter measurement intervals of 15 min[53]. This means that devices that can continuously assay blood glucose would be needed. More accurate and frequent blood glucose measurements can feed into automated and closed-loop glycemic control systems[54-62]. For instance, even when targeting a lower range of 4.4-8.3 mmol/L, one such system limited severe hypoglycemic episodes to only 0.01% of all blood glucose measurements and 0.8% of patients[59].

Table 5 Selected guideline recommendations

Casemix	Guideline (Year)	Recommended glycemic target range
Medical-Surgical	American Diabetes Association: Diabetes Care in the Hospital (2021) [47]	7.8-10 mmol/L. Lower targets may be appropriate for selected patients if they can be achieved without significant hypoglycemia
Post-CA	European Resuscitation Council and European Society of Intensive Care Medicine guidelines (2021)[48]	7.8-10 mmol/L
Sepsis	Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock (2016)[49]	< 10 mmol/L and avoid hypoglycemia. Lower targets may be appropriate for selected patients if they can be achieved without significant hypoglycemia
Surgical	WHO recommendations on intraoperative and postoperative measures for surgical site infection prevention: an evidence-based global perspective (2016)[50]	Unable to define target range, though glucose control protocols recommended
TBI	Brain Trauma Foundation's Guidelines for the Management of Severe Traumatic Brain Injury, Fourth Edition (2016)[51]	No recommendation

CA: Cardiac arrest; TBI: Traumatic brain injury; WHO: World Health Organization.

Optimization of glucose control protocols with respect to the following aspects may also be investigated: (1) Addition of bolus insulin "mid-protocol" during an insulin infusion to reduce peak insulin rates for insulin-resistant patients[63]; (2) transition of insulin administration route from intravenous to subcutaneous[64], and (3) use of DM-specific enteral formula for both DM and non-DM patients[65-67].

Given the influence of casemix on the optimal glycemic target range, further work may be done to personalize recommendations for various conditions[68]. For patients with DM, it remains unclear if the upper limit of blood glucose can be safely pushed beyond 10 mmol/L[69], given the risk of ketoacidosis or ketonemia[70]. To address this uncertainty, the LUCID trial will investigate if liberal blood glucose (target 10.0-14.0 mmol/L) will result in less incident hypoglycemia compared to usual care (target 6.0-10.0 mmol/L), while maintaining good clinical outcomes[71].

CONCLUSION

When choosing glycemic targets, one should keep the glycemic variability < 4 mmol/L and avoid targeting a lower limit of blood glucose < 4.4 mmol/L. The upper limit of blood glucose should be set according to casemix and the quality of glucose control. A lower glycemic target range (*i.e.*, blood glucose 4.5-7.8 mmol/L) would be favored for patients without diabetes mellitus, with traumatic brain injury, or who are at risk of surgical site infection. To avoid harm from hypoglycemia, strict adherence to glycemic control protocols and timely glucose measurements are required. In contrast, a higher glycemic target range (*i.e.*, blood glucose 7.8-10 mmol/L) would be favored as a default choice for medical-surgical patients and patients with diabetes mellitus. These targets may be modified if technical advances for blood glucose measurement and control can be achieved.

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MINIREVIEWS

Galectin-3 possible involvement in antipsychotic-induced metabolic changes of schizophrenia: A minireview

Milica M Borovcanin, Katarina Vesic, Milena Jovanovic, Natasa R Mijailovic

ORCID number: Milica M Borovcanin 0000-0002-2992-814X; Katarina Vesic 0000-0001-8861-4987; Milena Jovanovic 0000-0002-2026-2599; Natasa R Mijailovic 0000-0002-2125-0565.

Author contributions: Borovcanin MM presented the idea, structured the manuscript, incorporated all parts of the manuscript, and drew a figure; all authors have additionally searched the literature; Vesic K, Jovanovic M, and Mijailovic RN have given some new insights in specific fields of their competencies, and done the final revision of the manuscript and figure corrections; All authors have read, discussed, and approved the final version of the manuscript.

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Milica M Borovcanin, Department of Psychiatry, University of Kragujevac, Faculty of Medical Sciences, Kragujevac 34000, Sumadija, Serbia

Katarina Vesic, Department of Neurology, University of Kragujevac, Faculty of Medical Sciences, Kragujevac 34000, Sumadija, Serbia

Milena Jovanovic, PhD Studies, University of Kragujevac, Faculty of Medical Sciences, Kragujevac 34000, Sumadija, Serbia

Milena Jovanovic, Clinic for Nephrology and Dialysis, University Clinical Center Kragujevac, Kragujevac 34000, Sumadija, Serbia

Natasa R Mijailovic, Department of Pharmacy, University of Kragujevac, Faculty of Medical Sciences, Kragujevac 34000, Sumadija, Serbia

Corresponding author: Milica M Borovcanin, MD, PhD, Associate Professor, Department of Psychiatry, University of Kragujevac, Faculty of Medical Sciences, 69 Svetozara Markovica St, Kragujevac 34000, Sumadija, Serbia. milicaborovcanin@medf.kg.ac.rs

Abstract

Recently, specific immunometabolic profiles have been postulated in patients with schizophrenia, even before full-blown disease and independent of antipsychotic treatment. Proteomic profiling studies offer a promising potential for elucidating the cellular and molecular pathways that may be involved in the onset and progression of schizophrenia symptoms, and co-occurrent metabolic changes. In view of all this, we were intrigued to explore galectin-3 (Gal-3) as a glycan, and in our previous study, we measured its elevated levels in remission of schizophrenia. The finding may be a consequence of antipsychotic treatment and may have an impact on the onset of inflammation, the development of obesity, and the presumed cognitive changes in schizophrenia. In the animal study, it was shown that downregulation of Gal-3 was beneficial in insulin regulation of obesity and cognitive preservation. Strategies involving plasma exchange are discussed in this review, particularly in the context of Gal-3 elimination.

Key Words: Galectin-3; Schizophrenia; Metabolic syndrome; Insulin resistance; Cognition; Antipsychotics

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Core Tip: Atypical antipsychotic use can be associated with undesired metabolic effects. In that context, glycosylation has become a new target in the investigation of schizophrenia pathophysiology. As a glycan, galectin-3 (Gal-3) might be involved in the inflammation-insulin resistance-obesity cascade in schizophrenia, leading to cognitive changes. Eliminating Gal-3 influence may be beneficial in preserving cognition and reestablishing metabolic balance.

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INTRODUCTION

Clinical practice raises many questions regarding somatic states that accompany or are a consequence of mental illnesses. As schizophrenia is an extremely complex and debilitating mental disorder, overall treatment must take into account the somatic comorbidity of the patients. Although schizophrenia requires special attention and care in terms of lifestyle and antipsychotic treatment, a particular immunometabolic profile has recently been postulated, even before the disease onset[1]. The use of atypical antipsychotics is often associated with undesired metabolic and endocrine side effects including obesity, dyslipidemia, hyperglycemia, and insulin resistance[2]. To summarize, patients with schizophrenia most probably could have other comorbidities, regardless of their specific immunometabolic profile and antipsychotic therapy, and the somatic states may also lead to metabolic changes.

The identification of defects in cell biology and molecular phenotype underlying schizophrenia represents a challenging new approach to the study of this complex neurodegenerative disorder. Proteomic profiling studies, in which many proteins are tested for their relevance to the disease, are still in their infancy but the potential for elucidating the cellular and molecular pathways that may be involved in the onset and progression of schizophrenia is promising[3].

Altered protein post translational modifications such as glycosylation have become a new target of investigation in the pathophysiology of schizophrenia[4]. Glycosylation is an enzyme-mediated process in which a carbohydrate or carbohydrate structure, also referred to as a glycan, binds to a protein, lipid, or glycan substrate. Glycosylation is the most common and complex post translational modification and plays a critical role in protein-protein, protein-cell, and cell-cell interactions, including antibody binding, protein degradation, cellular endocytosis, and protease protection [5]. This process regulates nearly all cellular activities and has a critical role in the development and functioning of the central nervous system (CNS). Glycans are involved in many processes, such as neurite outgrowth and fasciculation, synapse formation and stabilization, modulation of synaptic efficacy, neurotransmission, and synaptic plasticity[6]. Altered glycosylation can significantly affect the properties of the glycosylated substrate, resulting in changes in its structure, localization, expression levels, molecular interactions, and/or substrate function.

Aberrant glycosylation has been identified in the serum, cerebrospinal fluid, urine, and postmortem brain tissue of schizophrenia patients[7]. Early evidence of glycosylation abnormalities in schizophrenia reported reduced glycoprotein expression in urine samples from male schizophrenia patients, and was consistent with abnormal glycan composition[8]. Altered monosaccharide composition of attached glycans was also found in the blood serum of the patients[9]. An increased serum glycoprotein level was also confirmed in young schizophrenia patients 13-17 years of age[10].

Abnormalities of N-linked glycosylation in schizophrenia have been observed in neurotransmitter receptor and transporter subunits, subunits from α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, kainate, and gamma-aminobutyric acid $(GABA)_A$ receptor families in various brain regions, including the dorsolateral prefrontal cortex, anterior cingulate cortex, and superior temporal gyrus[11-14]. Receptors containing abnormally N-glycosylated subunits have also been shown to

exhibit abnormal subcellular distribution in schizophrenia, suggesting cellular consequences of abnormal protein glycosylation[15]. Widespread glycosylation abnormalities due to abnormal glycosylation enzyme expression have also been reported in schizophrenia[16-18].

We have recently elaborated on the contrasting roles of the galectin-3 (Gal-3) through the schizophrenia continuance[19]. We also discussed the various somatic states co-occurring in schizophrenia that could be related to Gal-3. In this review, our interdisciplinary team seeks to further elucidate the mechanisms underlying the impact of glycans on early development, and how Gal-3 may further influence subsequent metabolic changes. However, our focus will be on the interplay of Gal-3 with antipsychotics during the course of the disease in an attempt to elucidate specific non-CNS systemic changes. Overall, that may lead to conclusions that allow more selective therapy of schizophrenia in the future.

GAL-3 AND NEURO-IMMUNO-METABOLIC CROSSTALK

In recent years, an increasing body of evidence has highlighted the involvement of Gal-3 in neurodevelopment and neurodegenerative diseases[20]. Scientific advances during the last decade have led to the discovery that Gal-3 plays a significant role in normal murine brain development, neuroblast migration, oligodendrocyte differentiation, and basal gliogenesis[21-24]. Chronic inflammation, mitochondrial damage and oxidative stress are factors common to neurodegenerative and metabolic diseases, in which sustained responses to inflammation contribute to neurodegeneration and progression of the disease[24,25]. Glial cell dysregulation is the main characteristic of chronic inflammation in neurodegenerative diseases, leading to changes in glycan expression in brain cells[26,27]. Previous studies have shown that inflammatory stimuli upregulate Gal-3 expression in activated microglia, and conversely, Gal-3 has been proposed as a modulator of the inflammatory response through microglial activation, cell adhesion, and cytokine release[28-32]. Recently, Gal-3 was shown to regulate microglial response to promote remyelination[23]. All this leads to the conclusion that Gal-3 is a key player in control of the switch between protective and disruptive microglial effects. In multiple sclerosis, Gal-3 expression is increased in periventricular inflammatory lesions[33]. Nishihara *et al*[34] investigated whether anti-Gal-3 antibodies might be a novel diagnostic marker and a possible therapeutic target in patients with secondary, progressive multiple sclerosis. Gal-3 deficiency reduces inflammation and disease severity in experimental autoimmune encephalomyelitis, Alzheimer's, and Parkinson's disease[35-37]. We reported elevated levels of Gal-3 in the stable phase of schizophrenia, with the suggestion that this glycan has a proinflammatory effect in the later phase[19] (Figure 1A). All the data indicate that Gal-3 might be a potential biomarker and therapeutic agent in this cohort of neurodegenerative disorders. Gal-3 is not only found in the cells themselves but is also secreted into the extracellular space in kidneys and heart, suggesting its multiple functions[38]. In addition to cell proliferation and differentiation, it promotes oxidative stress and proinflammatory processes and plays an important role in angiotensin II and aldosterone-induced myocardial and kidney fibrosis[39,40]. Studies have shown that elevated levels of Gal-3 are predictors of coronary disease in diabetes mellitus type 2 [41]. Gal-3 levels are elevated in maintenance hemodialysis patients, and can be used as a biomarker of vascular calcification, left ventricular hypertrophy, and left ventricular diastolic dysfunction[42-44].

Gal-3 has recently been recognized as an important modulator of biological functions and an emerging participant in the pathogenesis of immune/inflammatory and metabolic disorders[45-47] (Figure 1B). Gal-3 serum levels are elevated in women with polycystic ovary syndrome, especially those with insulin resistance, and those with increased insulin and glucose levels in the glucose tolerance test and it is considered a potential biomarker in prediabetes and diabetes[48-50]. The role of Gal-3 in metabolic disorders and the mechanism by which this lectin modulates excess fat mass, adipose tissue, systemic inflammation, and the associated impairment of glucose regulation, remains to be elucidated. Gal-3 is produced by many cell types, including adipocytes, and increased levels have been confirmed in obese patients[51,52]. Gal-3 is upregulated in growing adipose tissue and during inflammation[53,54]. Gal-3 is an important chemotactic factor for tissue macrophages in adipose tissue[55]. However, the role of Gal-3 in adipose tissue remains disputable because it exerts both deleterious and protective effects. In the general population, levels of circulating Gal-3 correlate positively with age, the prevalence of obesity, diabetes, hypercholesterolemia, and

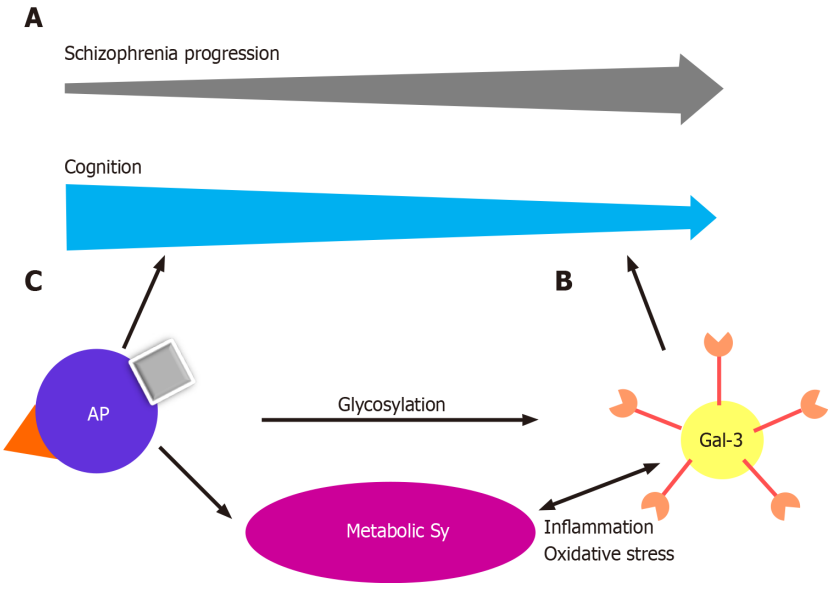

Figure 1 Galectin-3 and neuro-immuno-metabolic crosstalk. A and B: Considering the aspects of neuroprogression in schizophrenia, antipsychotics (APs) could have a beneficial role in improving cognitive functioning (A), but also, with galectin (Gal-3), could participate in undesired effects of immunometabolic disturbances (B); C: A potential cascade of metabolic syndrome (Sy) onset could be through the processes of glycosylation, Gal-3 elevation in the circulation, and secretion of proinflammatory cytokines, which individually and together could lead to cognitive deterioration.

hypertension, markers of inflammation, and target organ damage, indicating a clear association of Gal-3 with metabolic disorders and associated risk factors and complications[50,52,56,57]. Seemingly contradictory results were reported by Ohkura *et al*[58], who demonstrated that Gal-3 affected the concentration of insulin more than that of glucose, and that the increase of Gal-3 activity in diabetic patients had a protective effect on insulin resistance.

Obesity may influence not only behavior, cognition, and mood, but also adipose tissue dysfunction and inflammation, trigger impairment of insulin signaling, compromise the storage of triglycerides, and contribute to insulin resistance with high levels of free fatty acids[59]. Moreover, all the processes associated with insulin resistance and chronic hyperglycemia induce oxidative stress and inflammatory responses that lead to neuronal death, cognitive impairment, and neurodegeneration.

Hippocampal insulin resistance is the key factor in cognitive deficits. In an animal model study, insulin signaling in the hippocampus was shown to be affected by a cascade in which obesity induced chronic inflammation and chronic inflammation had role in obesity-related insulin resistance[60]. Moreover, chronic inflammation is suppressed by Gal-3, so Gal-3 directly impacts insulin signaling and might be a targetable link between inflammation and insulin sensitivity. Qin *et al*[60] suggested that the development of cognitive deficits in obese people could be inhibited through Gal-3 decrement.

Obesity is reported in approximately 50% of patients, metabolic syndrome in up to 40%, glucose intolerance in up to 25%, and diabetes in up to 15% of patients with schizophrenia[61]. The increased prevalence of these conditions is multifactorial. Antipsychotics can cause weight gain, glucose intolerance, and other metabolic complications[62] (Figure 1C). A recent meta-analysis of metabolic parameters in patients with first-episode psychosis, which can be described as early schizophrenia, showed increased insulin resistance and impaired glucose tolerance in the patients compared with healthy, matched controls, implying that schizophrenia might share intrinsic inflammatory disease pathways with type 2 diabetes[63]. We have previously discussed our findings of the possibly protective properties of Gal-3 in type-2 diabetes, but triggering metabolic changes and myocardial fibrosis[19].

GAL-3 AND ANTIPSYCHOTIC TREATMENT IN SCHIZOPHRENIA

Relatively few studies have investigated the effects of antipsychotic treatment on the serum glycosylation profiles in schizophrenia patients. Reports examining glycan expression in schizophrenia patients showed that the glycan profile in serum and

cerebrospinal fluid of first onset, unmedicated schizophrenia patients differs from the profile of healthy controls[64]. The results showed that some types of sialylated N-glycans derived from low-abundance serum proteins are significantly increased in patients with schizophrenia compared with controls. The study found a two-fold increase in serum glycan levels in male schizophrenia patients, with gender-specific differences also apparent[65]. Glycemic differences have also been reported in patients with acute paranoid schizophrenia before and after 6 wk of treatment with olanzapine, an atypical antipsychotic medication[65]. Olanzapine administration increased galactosylation and sialylation of serum N-glycans, suggesting increased activity of specific galactosyltransferases and increased availability of galactose residues for sialylation. The results indicate that the glycosylation profile of serum proteins can be used to monitor patients with schizophrenia after treatment. Given the confirmed effects of olanzapine on hepatic enzymes, it is possible that the reported changes in glycosylation induced by olanzapine treatment may occur because of the altered activity of hepatic glycosylation-processing enzymes[66].

As schizophrenia may have an evolving, progressive pathology, Narayan *et al*[67] focused on changes in gene expression and molecular pathways throughout illness progression. They assessed the alterations in patients treated with the typical antipsychotic medication, chlorpromazine, at early (≤ 4 years), intermediate (7-18 years), and late (≥ 28 years) stages of schizophrenia. The results showed that biopolymer glycosylation, protein amino acid glycosylation, and glycoprotein biosynthesis were increased in intermediate-stage patients. Analysis of differences in gene expression revealed that carbohydrate metabolism was dominant in short-term illness, whereas lipid metabolism prevailed in intermediate-term illness. Overall, short-term illness was particularly associated with disruptions in gene expression, metal ion binding, ribonucleic acid processing, and vesicle-mediated transport. Considerably different from short-term illness, long-term illness was associated with inflammation, glycosylation, apoptosis, and immune dysfunction.

A postmortem study compared the effects of atypical (olanzapine and risperidone) *vs* typical antipsychotics (chlorpromazine and haloperidol) on the livers, various genes, and molecular functions of patients[68]. The results demonstrated that typical antipsychotics affected genes associated with nuclear protein, stress responses, and phosphorylation, whereas atypical antipsychotics increased gene expression associated with Golgi/endoplasmic reticulum, and cytoplasmic transport, suggesting that atypical antipsychotics affect post translational modifications. The study showed that olanzapine treatment increased the expression of the *B4GALT1* gene in the liver of schizophrenia patients. That gene encodes β1,4-galactosyltransferase I (Gal-T1). Increased expression and activity of the enzyme lead to increased galactosylation of GlcNAc residues in glycans, which is consistent with the results of a study performed by Telford *et al*[65]. Genes associated with lipid metabolism were consistently downregulated in the typical compared with the atypical antipsychotic group.

However, dysregulation of adipose tissue homeostasis appears to be a critical factor [69]. An untargeted proteomic analysis of the effect of antipsychotics on adipose tissue was performed in a rat schizophrenia-like methylazoxymethanol acetate model[70]. Chronic, 8-wk-long application of three antipsychotics was characterized by differences in the likelihood of inducing metabolic alterations. Olanzapine, risperidone, and haloperidol, caused alterations in protein N-linked glycosylation in adipose tissue, providing further evidence that dysregulated glycosylation in schizophrenia may also be caused to some extent by antipsychotic treatment. Drug-specific effects included upregulation of insulin resistance (olanzapine), upregulation of fatty acid metabolism (risperidone), and upregulation of nucleic acid metabolism (haloperidol). Individual metabolic characteristics might also predispose to a different likelihood of becoming obese after antipsychotic treatment. Gal-3 has been shown to be associated with the onset of schizophrenia, and its elevation could have consequent deleterious effects (Figure 1). In addition, it must be taken into account that our patients were treated with risperidone or paliperidone, which are antipsychotics that may upregulate fatty acid metabolism and have Gal-3-elevating properties[71].

CONCLUSION

In this context, it is necessary and urgent to develop more selective treatment strategies. The phase of the illness also needs to be considered, with a focus on early interventions. The possibility that schizophrenia is secondary to a circulating, large molecular-weight substance has been explored with variable success. However, a

double-blind evaluation of plasmapheresis in ten patients with schizophrenia yielded negative results, and the procedure did not lead to a reduction in psychosis[72]. As hypercholesterolemia has been treated with plasmapheresis, and recently the therapeutic usefulness of Gal-3 depletion apheresis has been demonstrated in inflammation-mediated disease, targeting Gal-3 molecule may be a useful way to address immunometabolic problems and cognitive deterioration in schizophrenia in the future [73,74].

The question is whether extrapolations of preclinical and research data are applicable in clinical practice. Gal-3 relevance could be very interesting in further exploration of the genesis of schizophrenia in parallel with the metabolic alterations of the patients. It might be useful for clinicians to become familiar with this molecule and its precise roles in each phase of the disease in order to improve cognition and reestablishing metabolic balance in schizophrenia.

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ORIGINAL ARTICLE

Basic Study

Medication adherence and quality of life among type-2 diabetes mellitus patients in India

Rakhi Mishra, Suresh K Sharma, Rajni Verma, Priyanka Kangra, Preeti Dahiya, Preeti Kumari, Priya Sahu, Priyanka Bhakar, Reena Kumawat, Ravinder Kaur, Ravinder Kaur, Ravi Kant

ORCID number: Rakhi Mishra 0000-0003-1400-8196; Suresh K Sharma 0000-0003-1214-8865; Rajni Verma 0000-0002-0475-3507; Priyanka Kangra 0000-0003-1926-5394; Preeti Dahiya 0000-0003-1261-8652; Preeti Kumari 0000-0001-5857-8698; Priya Sahu 0000-0001-8526-1119; Priyanka Bhakar 0000-0002-9910-658X; Reena Kumawat 0000-0002-5248-1817; Ravinder Kaur 0000-0003-4912-7371; Ravinder Kaur 0000-0002-9397-7472; Ravi Kant 0000-0003-1144-4478.

Author contributions: Mishra R, Sharma SK, Kant R designed and coordinated the study; Verma R, Kangra P, Dahiya P, Kumari P, Sahu P, Bhakar P, Kumawat R, Kaur R, Kaur R acquired and analyzed data; Mishra R, Verma R, Kangra P, Dahiya P, Kumari P, Sahu P, Bhakar P, Kumawat R, Kaur R, Kaur R interpreted the data; Mishra R, Sharma SK, Kant R wrote the manuscript; All authors approved the final version of the article.

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Rakhi Mishra, Suresh K Sharma, Rajni Verma, Priyanka Kangra, Preeti Dahiya, Preeti Kumari, Priya Sahu, Priyanka Bhakar, Reena Kumawat, Ravinder Kaur, Ravinder Kaur, College of Nursing, All India Institute of Medical Sciences, Uttarakhand 249203, Rishikesh, India

Ravi Kant, Department of Medicine, All India Institute of Medical Sciences, Uttarakhand 249203, Rishikesh, India

Corresponding author: Rakhi Mishra, MSc, Assistant Professor, College of Nursing, All India Institute of Medical Sciences, Virbhadra Road Shivaji Nagar, Near Barrage, Uttarakhand 249203, Rishikesh, India. mishrarakhi92@gmail.com

Abstract

BACKGROUND

Diabetes mellitus (DM) is a progressively increasing metabolic disorder and a significant public health burden that demands immediate global attention. However, there is a paucity of data about adherence to antidiabetic drugs among patients with type-2 (T2)DM in Uttarakhand, India. Outpatient research reported that more than 50% of patients do not adhere to the correct administration and appropriate medicine dosage. It has been reported that patients with chronic diseases who adhere to treatment may experience improvement in quality of life (QoL) and vice versa.

AIM

To assess the adherence to antidiabetic medication and QoL among patients with T2DM.

METHODS

This cross-sectional descriptive study was conducted at a tertiary care hospital in Uttarakhand, India. The Medication Adherence Rating Scale and World Health Organization QoL-BREF scale were used to assess medication adherence and QoL.

RESULTS

Two hundred seventy-seven patients suffering from T2DM participated in the study. Their mean age was 50.80 (± 10.6) years, 155 (56%) had a poor adherence level and 122 (44%) had a good adherence level to antidiabetic medications. After adjusting for sociodemographic factors, multiple linear regression analysis found

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patients who were adherent to antidiabetic medications had significantly higher mean overall perception of QoL and overall perception of health, with beta scores of 0.36 and 0.34, respectively (both P = 0.000) points compared with nonadherent patients.

CONCLUSION

There was an association between medication adherence and QoL in patients with T2DM. Hence, there is a need to plan awareness and counseling programs followed by regular follow-up to motivate patient adherence to recommended treatment and lifestyle regimens.

Key Words: Medication adherence; Quality of life; Diabetes mellitus; Tertiary care hospital; India

Core Tip: Many research articles have been published on the epidemiology, complications, therapies, comparisons of treatments, and healthcare strategies for diabetes mellitus (DM). The literature shows that patient adherence to antidiabetic medications and quality of life (QoL) are interrelated. Patients with diabetes who adhere to their treatment can experience an improvement in QoL and vice versa. This study focused on (1) adherence to antidiabetic medication and QoL among T2DM patients; (2) finding the relationship between adherence to antidiabetic medication and QoL; and (3) determining the association between adherence to antidiabetic medications and QoL and selected demographic variables.

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INTRODUCTION

Diabetes mellitus (DM) is a progressively increasing metabolic disorder that has become a significant public health burden. The World Health Organization has identified DM as an important noncommunicable diseases that demands immediate global attention[1]. The International Diabetes Federation has reported that 463 million adults between 20-79 years of age are living with diabetes and that the total will rise to 700 million by 2045[2]. This chronic disorder is rated among the top ten causes of death (4.2 million) globally and has attained pandemic proportions worldwide[2]. In addition to increased mortality, diabetes can lead to poor physical and mental health. Moreover, problems like increased blood glucose level and dietary and exercise limitations demand repeated insulin injections. However, musculoskeletal and vascular complication negatively affect the quality of life (QoL) of patients with DM [3]. To prevent the development of fatal complications associated with DM, glycemic control is required. To achieve that goal, it is necessary to encourage patients to adhere to therapeutic regimens, change their life style, and follow the recommendations of their clinicians[4]. Studies have shown that patient adherence to chronic-disease treatment is low[5]. Research involving outpatients reported that more than 50% do not adhere to the correct medicine administration and dosage[6]. The diabetes literature shows that patient QoL and medication adherence are interrelated. It has been reported that patients with chronic diseases who adhere to their treatment may experience improvement in QoL and vice versa[7]. However, there is a paucity of data about adherence to antidiabetic drugs among patients with T2DM, especially in Uttarakhand. Therefore, this study was conducted to assess the adherence to antidiabetic medication and QoL by patients with T2DM.

MATERIALS AND METHODS

Participants and settings

This cross-sectional descriptive study was conducted at a tertiary care hospital in Uttarakhand, India to assess adherence to antidiabetic medications and QoL by patients with T2DM. A sample size of 350 was calculated considering that an estimated 31.2% of the population would be nonadherent to antidiabetic medications, a margin of error of 5% with a 95% confidence interval (CI), a 10% study dropout rate, and entering potential confounders as covariates in the regression model[8,9]. Patients between 21 and 75 years of age who were diagnosed with T2DM and visited the outpatient department between February 10, 2020 and March 19, 2020 were eligible for inclusion. Those who were under treatment for T2DM for less than 6 mo or had cognitive and neurological impairment were excluded.

Ethical approval

Ethical permission was obtained from institution ethics committee, vide letter no. 368/IEC/STS/2019. Participants were informed about the purpose of research and ensured about anonymity and confidentiality of the information. A written informed voluntary participation consent was obtained from each study participant.

Instruments

Participant data were collected with a structured interview questionnaire that included sociodemographic characteristics (section I), the Medication Adherence Rating Scale (MARS, section II), and the WHOQoL-BREF scale (section III). Questionnaires were administered in the Hindi Language, which is the national language of India. MARS is a 10-item questionnaire with validated validity, and developed originally in English[10]. To avoid acquiescence bias, the items in the scale have a dichotomous response (yes/no). The minimum score was 0 and the maximum score was 10. The summed total score was categorized as non- or poor adherence (0-5) or adherence or good adherence (6-10). The WHOQoL-BREF is a generic instrument developed to measure QoL of patients suffering from T2DM by the WHO criteria and is a short version of the 100 item WHOQoL-100[11]. The WHOQoL-BREF consists of 26 items divided into four QoL domains, Physical health (seven items), psychological health (six items), social relationships (three items), and environmental health (eight items). The two remaining questions assessed an individual's overall perception of QoL and overall perception of health. All the questions in the instrument are scaled in a positive direction from 1 to 5, with a high score indicating good QoL, except for items three, four, and 26. The domain score was calculated from the mean score of all items within each domain. To make the domain scores comparable to WHOQoL-100 scores, the calculated mean scores were multiplied by 4[11]. Permission was obtained to use the MARS and WHOQoL-BREF tools, and a license agreement was signed by the appropriate authority. The validity and reliability of the tools were pre-established using Cronbach's alpha, $r = 0.70$ for the WHOQoL-BREF and $r = 0.75$ for MARS[10,12]. A standardized pilot-tested collection form was used to collect data from participants. The average time taken to complete one interview was around 20 min.

Statistical analysis

Data were coded and then entered onto Excel worksheets. The Statistical Package for the Social Sciences (SPSS 21.0) was used for statistical analysis. Descriptive and inferential statistics were used for data analysis. Sociodemographic characteristics were reported as frequencies (*n*) and percentages (%). Adherence to antidiabetic medication- and health-related QoL was reported as means and standard deviation (SD). Multiple linear regression analysis was performed to assess the effect of adherence to antidiabetic medication within each QoL domain after adjusting the estimates for some sociodemographic variables. A P value of < 0.05 was considered statistically significant.

RESULTS

A total of 277 patients with T2DM were recruited during the study period. The mean ± SD age was 50.80 ± 10.6 years. More than half of the patients were men (57%), residing in urban area (63.9%) with a distance of more than 10 km from hospital (66.1%), having joint family (65.3%), and suffering from T2DM for 1-5 years (54.2%). Nearly

one-third (32.9%) had an educational status up to the primary level. The majority had associated comorbidities, including thyroid (54.5%) and hypertension (41.5%). The clinical histories revealed that 47.3% were taking both insulin and oral hypoglycemic agents (OHAs) for treatment along with lifestyle modifications (Tables 1 and 2).

Of the 277 patients included in this study, 155 (56%) had poor adherence scores of 0-5 and 122 (44%) had good adherence scores of 6-10) for antidiabetic medications. The mean overall perception of QoL and health scores were 68.16 ± 14.69 and 63.97 ± 16.51 respectively. Tables 1 and 2 shows the mean scores of the four domains stratified by sociodemographic and clinical characteristics and the two individual questions assessing the overall perception of QoL and health. Higher mean QoL score were reported by those with postgraduate and above education, incomes > 30001 INR, residing < 5 km from the hospital, suffering with T2DM for more than 15 years, and on glimepiride.

Multiple regression analysis found that medication adherence was an independent predictor of QoL ($P < 0.05$) in the patients in this study after adjusting for various sociodemographic characteristics including age, marital status, educational qualification, type of family, and monthly income. Patients who were adherent to antidiabetic medications had significantly higher mean overall perception of QoL and health scores, ($P = 0.000$) compared with nonadherent patients. Their beta scores of 0.36 and 0.34 points, respectively (Table 3).

DISCUSSION

DM is a chronic disease that requires patients to be on long-term drug therapy. Poor treatment adherence and lifestyle habits are significant barriers in the treatment of DM. The primary objective of diabetes management is to improve patient health-related QoL which is now a growing area of interest and has emerged as a significant chronic-disease outcome. In developed nations, approximately 50% of diabetes patients do not adhere to the recommended therapies[13]. The literature has shown that medication adherence is associated with improved disease control[6]. This study was conducted to assess adherence to antidiabetic medications and QoL in patients with T2DM attending the outpatient department of a tertiary care hospital. Medication adherence is a key factor because it is directly related to the disease outcome. However, nonadherence may alter all QoL dimensions. This study found that 155 of the participants (56%) were nonadherent and 122 (44%) were adherent to antidiabetic medications. Worldwide studies using various research assessment instruments and systematic reviews have addressed issues of poor medication adherence by diabetes patients[14]. Our findings are similar to those of Ahmad *et al*[15], who reported that 53% of their respondents were nonadherent to medications. However, much lower rates of nonadherence have been seen in studies conducted by Bagonza *et al*[16], Pascal *et al*[17], and Elsous *et al*[18] who reported rates between 16.7%% and 42%. The difference in adherence might be explained by variations in healthcare services, socioeconomic status, and the metrics used for assessment of adherence across the study settings. However, a study conducted in Oman reported overall good patient adherence to the medication regimen (80%), which is higher than our finding[19]. A study in 129 patients by Fadare *et al*[20] reported 40.6% good, 32.8% medium, and 26.6% poor adherence to medication regimens.

This study found a statistically significant relation ($P < 0.05$) between age and monthly income and good adherence to antidiabetic medications. Similarly, Gelaw *et al*[21] reported that increased age was significantly associated ($P < 0.05$) with good adherence to treatment. It is expected that patients with high education levels would have better adherence to medication regimen, and that was confirmed by Ahmed *et al* [22], who found that patients with graduate-level educations were highly adherent. Contrary to our findings, Fadare *et al*[20] did not find significant differences in adherence ($P < 0.05$) between levels of education and adherence. Our findings are similar to those of Gelaw *et al*[21] who reported that married patients had a higher rate of therapeutic adherence (48.6%) than single, widowed, or divorced patients, but Khan *et al*[23] did find a significant impact of marital status on patient adherence.

Gelaw *et al*[21] reported that 82.07% of patients with a duration of diabetes ≤ 5 years were more compliant to medication than those with diabetes for > 5 years, and the difference was statistically significant. Similar to our finding that 155 (56%) of patients were nonadherent to antidiabetic medications, Bezie *et al*[24] reported that patients who had been on diabetic treatment for < 5 years were poorly adherent to treatment. It is likely that patients who have been on treatment for a short duration are less aware

Table 1 Quality of life scores, sociodemographic characteristics, and medication adherence of the study participants

Demographic variables	*n* (%)	QoL domains, mean ± SD				Overall perception on QoL	Overall perception of Health
		Physical domain	Psychological domain	Social domain	Environmental domain		
Age, yr							
21-46	77 (27.8)	59.67 ± 15.88	53.12 ± 17.76	69.97 ± 12.07	55.14 ± 11.68	67.79 ± 15.61	64.45 ± 16.74
47-71	193 (69.7)	56.29 ± 15.03	52.64 ± 14.35	69.98 ± 14.66	57.99 ± 11.95	68.50 ± 14.23	63.73 ± 16.28
< 71	07 (2.50)	41.86 ± 10.73	44.71 ± 18.98	62.57 ± 21.75	56.28 ± 13.35	62.86 ± 17.99	54.29 ± 19.02
Gender							
Male	158 (57)	57.30 ± 14.93	53.59 ± 14.48	70.33 ± 14.72	58.02 ± 11.32	69.24 ± 13.66	66.33 ± 15.82
Female	119 (43)	56.29 ± 16.05	51.23 ± 16.68	69.07 ± 13.47	56.01 ± 12.65	66.72 ± 15.89	60.84 ± 16.95
Marital status							
Married	254 (91.7)	57.09 ± 15.63	52.82 ± 15.39	70.36 ± 12.75	57.13 ± 11.95	68.35 ± 14.86	64.33 ± 16.64
Single	23 (8.30)	54.48 ± 12.61	49.83 ± 16.48	63.52 ± 24.72	57.48 ± 11.95	66.09 ± 12.70	60.00 ± 14.77
Educational qualification							
Illiterate	51 (18.4)	54.18 ± 17.35	49.18 ± 16.08	67.72 ± 11.86	53.65 ± 11.05	64.31 ± 15.13	59.22 ± 16.95
Primary school	91 (32.9)	55.91 ± 16.04	51.32 ± 15.83	67.91 ± 14.39	54.15 ± 12.10	67.03 ± 15.88	62.86 ± 18.27
Secondary school	71 (25.6)	58.17 ± 15.86	54.83 ± 14.78	72.19 ± 14.39	58.98 ± 10.34	69.30 ± 13.87	65.92 ± 14.89
Graduate school and above	64 (23.1)	58.94 ± 11.82	54.56 ± 14.90	71.45 ± 16.72	62.19 ± 12.08	71.56 ± 12.75	67.19 ± 14.42
Type of family							
Nuclear	96 (34.7)	60.42 ± 16.10	56.05 ± 15.51	71.03 ± 13.18	58.26 ± 11.21	68.54 ± 15.01	65.00 ± 16.42
Joint	181 (65.3)	54.99 ± 14.72	50.73 ± 15.18	69.14 ± 14.69	56.57 ± 12.29	67.96 ± 14.56	63.43 ± 16.58
Monthly income, INR							
< 20000	127 (45.8)	54.20 ± 15.40	51.24 ± 15.48	67.58 ± 14.86	54.59 ± 12.14	66.61 ± 15.54	60.94 ± 16.88
20000-30000	91 (32.9)	56.37 ± 15.68	52.77 ± 15.56	69.77 ± 14.57	57.77 ± 11.44	67.69 ± 13.91	63.74 ± 15.47
> 30001	59 (21.3)	63.39 ± 13.14	55.13 ± 15.28	74.59 ± 10.69	61.71 ± 10.87	72.20 ± 13.40	70.85 ± 15.46
Residence							
Urban	177 (63.9)	59.80 ± 15.25	54.29 ± 15.79	71.48 ± 12.61	57.60 ± 12.09	69.04 ± 14.29	66.10 ± 15.63
Rural	100 (36.1)	51.68 ± 14.32	49.54 ± 14.49	66.80 ± 16.26	56.36 ± 11.66	66.60 ± 15.32	60.20 ± 17.41

Adherence level							
Nonadherent	155 (55.96)	52.32 ± 14.59	48.94 ± 14.56	69.73 ± 13.91	56.17 ± 12.32	64.90 ± 15.35	60.65 ± 16.50
Adherent	122 (44.04)	62.65 ± 14.48	57.19 ± 15.44	69.87 ± 14.59	58.40 ± 11.35	72.30 ± 12.71	68.20 ± 15.59

Data are *n* (%) or mean ± SD. INR: Indian rupee; QoL: Quality of life.

Table 2 Quality of life scores and clinical characteristics of the study participants

Clinical characteristics	*n* (%)277	Physical domain	Psychological domain	Social domain	Environmental domain	Overall perception of QoL	Overall perception of health
Duration of T2DM, yr							
1-5	150 (54.2)	57.19 ± 14.61	52.77 ± 15.54	70.98 ± 12.06	57.03 ± 12.39	68.67 ± 14.91	64.13 ± 15.77
6-10	90 (32.5)	54.98 ± 14.91	51.07 ± 14.11	67.74 ± 17.02	57.12 ± 12.21	66.67 ± 14.06	63.78 ± 16.93
11-15	31 (11.2)	60.35 ± 19.86	56.06 ± 18.94	70.42 ± 14.96	57.16 ± 10.03	69.03 ± 15.35	63.23 ± 19.39
> 15	06 (2.2)	59.33 ± 15.86	52.17 ± 14.69	67.67 ± 12.13	60.67 ± 03.61	73.33 ± 16.33	66.67 ± 16.33
Drug used							
Metformin	80 (28.9)	56.27 ± 14.55	51.00 ± 13.68	69.77 ± 14.09	56.46 ± 11.91	67.75 ± 13.31	64.25 ± 15.49
Glimepiride	66 (23.8)	61.20 ± 15.54	56.64 ± 15.21	71.44 ± 12.32	56.38 ± 12.10	71.82 ± 14.45	66.36 ± 16.14
Other OHA	131 (47.3)	55.05 ± 15.53	51.49 ± 16.37	68.98 ± 15.12	57.97 ± 11.90	66.56 ± 15.38	62.60 ± 17.26
Chronic comorbid illness							
Hypertension	115 (41.5)	56.31 ± 15.46	51.94 ± 15.54	67.27 ± 15.44	56.81 ± 10.94	65.91 ± 14.74	61.74 ± 17.28
Thyroid	151 (54.5)	57.62 ± 15.48	53.54 ± 15.01	71.87 ± 13.11	57.39 ± 12.87	69.93 ± 14.21	65.70 ± 15.89
CAD	11 (4.00)	52.36 ± 13.91	46.00 ± 20.00	67.64 ± 10.76	57.54 ± 08.85	67.27 ± 18.49	63.64 ± 15.01

CAD: Coronary artery disease; DM: Diabetes mellitus; OHA: Oral hypoglycemic agent, QoL: Quality of life; T2DM: Type-2 diabetes mellitus. Data are *n* (%) or mean ± SD.

of the disease condition and are thereby nonadherent to the antidiabetic medications. However, patients with a longer disease duration are likely to have had more contacts with their healthcare providers, may have a better understanding of their regimen, are more likely to be self-motivated to take prescribed medications. However, the association of treatment adherence and the number of years with diabetes was not statistically significant.

Table 3 Multiple linear regression analysis of predicting variables for quality of life domains

Demographic variable	Physical domain		Psychological domain		Social domain		Environmental domain		Overall perception of QoL		Overall perception of health	
	β	*P* value	*β*	*P* value	*β*	*P* value	*β*	*P* value	*β*	*P* value	*β*	*P* value
Age, (ref ≤ 71 yr)	-4.84	0.009	-1.12	0.56	-0.28	0.87	2.62	0.08	0.04	0.65	-0.15	0.14
Gender (ref = male)	-0.42	0.83	-1.48	0.47	2.38	0.21	1.52	0.34	-0.003	0.98	-0.18	0.09
Marital status (ref = single)	-1.13	0.47	-1.77	0.29	-5.98	0	-0.24	0.85	-0.04	0.64	-0.07	0.41
Educational qualification (ref = illiterate)	-0.04	0.96	1.27	0.19	0.92	0.3	2.75	0	0.09	0.06	0.03	0.5
Type of family (ref = nuclear)	-2.96	0.08	-4.32	0.02	-1.45	0.37	-2.03	0.14	-0.03	0.71	-0.02	0.84
Monthly income (ref ≤ 20,000)	3.57	0.005	0.35	0.79	2.86	0.02	2.13	0.04	0.05	0.39	0.16	0.02
Habitat (ref = rural)	-3.37	0.06	-0.57	0.76	-1.88	0.27	1.57	0.21	-0.02	0.82	-0.11	0.26
Years with DM (ref ≥ 15)	0.75	0.5	0.27	0.82	-1.26	0.31	-0.12	0.89	0	0.99	0.004	0.96
Drug used (ref = other OHA)	-1.84	0.07	-0.29	0.79	-0.74	0.46	0.52	0.54	-0.06	0.28	-0.09	0.12
Chronic comorbid illness (ref = hypertension)	-0.73	0.63	-0.4	0.8	2.47	0.09	0.58	0.64	0.13	0.08	0.07	0.41
Adherent level (ref = nonadherent)	8.91	0	7.59	0	-0.55	0.74	1.72	0.22	0.36	0	0.34	0

β-Standardized regression coefficient; DM: Diabetes mellitus; OHA: Oral hypoglycemic agent; QoL: Quality of Life; Ref: Reference group.

Participants who do not have diabetes-related complications had a high level of adherence. In contrast, poor medication adherence may result in comorbidities. Participants with no diabetes-related complications requires fewer medications. Thus, their adherence to antidiabetic medication might increase, and the risk of developing comorbidities might decrease[25]. This study did not find a significant association between comorbidities and the level of adherence. Considering the multifactorial nature of poor medication adherence, it is understood that only a sustained, coordinated effort will ensure optimal medication adherence. This study has clearly shown that diabetes impaired the physical and psychological QoL domain. QoL measurements should thus become routine in the clinical management of diabetic patients. Another factor which was found to be positively associated with adherence was knowledge of DM and its medications. Keeping in mind the high prevalence of both diabetes and nonadherence to the treatment regimen, additional nurses should be trained to run special diabetic clinics at rates that patients can easily afford to pay. Creating awareness and educating the patients regarding the disease and its management will definitely help to improve the adherence level and QoL.

CONCLUSION

This study found that more than 50% of the participants were nonadherent to antidiabetic medications and that QoL scores were associated with the level of patient adherence. There is a need to plan and implement awareness and counseling programs and regular follow-up to motivate patients to improve adherence to recommended treatment and lifestyle regimens.

Scope for future work

The study was limited by a small patient sample, but the findings can be expanded by machine-learning analysis and statistical methods designed to extract information from large data samples. Specifically, random forest algorithms and artificial neural networks can be used to determine which predictors are more important for the prediction of medication adherence or quality of life (QoL).

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ARTICLE HIGHLIGHTS

Research background

Diabetes mellitus (DM) is a progressive metabolic disorder that has become a significant public health burden that demands immediate global attention. However, there is a paucity of data on adherence to antidiabetic drugs by patients with type-two (T2)DM in Uttarakhand, India. Current research in outpatients has shown that more than 50% of patients do not adhere to the correct administration and appropriate dosage of antidiabetic medications. It has been reported that patients with chronic diseases who adhere to treatment may experience improvement in quality of life (QoL) and vice versa.

Research motivation

DM is a progressively increasing metabolic disorder that has become a significant public health burden and demands immediate global attention. The paucity of data on adherence to antidiabetic drugs by patients with T2DM in Uttarakhand, India prompted this study.

Research objectives

The study was conducted to assess the adherence to antidiabetic medications and QoL in patients with T2DM.

Research methods

This cross-sectional descriptive study was conducted at a tertiary care hospital in Uttarakhand, India. The Medication Adherence Rating Scale and World Health Organization QoL-BREF scale were used to assess medication adherence and QoL.

Research results

A group of 277 outpatients with T2DM participated in the study. Their mean age was 50.80 ± 10.6 years, 155 (56%) had poor, and 122 (44%) had good antidiabetic medication adherence. After adjusting for sociodemographic variables, multiple linear regression analysis found that patients who were adherent to antidiabetic medications had significantly a higher overall mean perception of QoL and health, with beta scores of 0.36 and 0.34 points, respectively (both $P = 0.000$) compared with nonadherent patients.

Research conclusions

Adherence to medications by patients with T2DM was correlated with QoL. Hence, there is a need to plan and implement awareness and counseling programs followed by regular follow-up to motivate patient adherence to recommended treatment and lifestyle regimens.

Research perspectives

Many research articles have been published about the epidemiology, complications, therapies, comparisons of treatments, and healthcare strategies for DM. The literature shows that patient adherence to antidiabetic medications and QoL are interrelated. Patients with diabetes who adhere to their treatment can experience an improvement in QoL and vice versa. This study focused on (1) adherence to antidiabetic medications and QoL in T2DM; (2) finding the relationship between adherence to antidiabetic medications and QoL; and (3) determining the association between adherence to antidiabetic medications and QoL and selected demographic variable.

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ORIGINAL ARTICLE

Basic Study

Metabolic and inflammatory functions of cannabinoid receptor type 1 are differentially modulated by adiponectin

Qiong Wei, Jong Han Lee, Chia-Shan Wu, Qun S Zang, Shaodong Guo, Hui-Chen Lu, Yuxiang Sun

ORCID number: Qiong Wei 0000-0001-6836-9929; Jong Han Lee 0000-0002-5614-1537; Chia-Shan Wu 0000-0002-6034-939X; Qun S Zang 0000-0002-6397-8896; Shaodong Guo 0000-0001-5126-731X; Hui-Chen Lu 0000-0002-6628-7177; Yuxiang Sun 0000-0002-6656-3733.

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Qiong Wei, Department of Endocrinology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing 210009, Jiangsu Province, China

Qiong Wei, Jong Han Lee, Chia-Shan Wu, Yuxiang Sun, Department of Pediatrics, USDA/ARS Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX 77030, United States

Jong Han Lee, Department of Marine Bioindustry, Hanseo University, Seosan 31962, South Korea

Chia-Shan Wu, Shaodong Guo, Yuxiang Sun, Department of Nutrition, Texas A and M University, College Station, TX 7743, United States

Qun S Zang, Department of Surgery, Stritch School of Medicine, Loyola University Chicago Health Science Campus, Maywood, IL 60153, United States

Hui-Chen Lu, Department of Psychological and Brain Sciences, Linda and Jack Gill Center of for Biomolecular Science, Bloomington, IN 47405, United States

Corresponding author: Yuxiang Sun, MD, PhD, Associate Professor, Department of Nutrition, Texas A and M University, 214C Cater-Mattil, 2253 TAMU College Station, TX 7743, United States. yuxiang.sun@tamu.edu

Abstract

BACKGROUND

Antagonists of cannabinoid type 1 receptor (*CB1*) have been shown to promote body weight loss and improve insulin sensitivity. Cannabinoids decrease adiponectin, and *CB1* blocker increase adiponectin. However, the mediators of *CB1* actions are not well defined.

AIM

To investigate whether the beneficial effects of *CB1* inhibition are, at least in part, mediated by adiponectin.

METHODS

We compared metabolic and inflammatory phenotypes of wild-type (WT) mice, *CB1*-null (*CB1*$^{-/-}$) and *CB1*/adiponectin double-knockout (DKO) mice. We assessed the insulin sensitivity using insulin tolerance test and glucose tolerance test, and inflammation using flow cytometry analysis of macrophages.

RESULTS

CB1$^{-/-}$ mice exhibited significantly reduced body weight and fat mass when compared to WT mice. While no significance was found in total daily food intake and locomotor activity, *CB1*$^{-/-}$ mice showed increased energy expenditure, enhanced thermogenesis in brown adipose tissue (BAT), and improved insulin sensitivity compared to WT mice. DKO showed no difference in body weight, adiposity, nor insulin sensitivity; only showed a modestly elevated thermogenesis in BAT compared to *CB1*$^{-/-}$ mice. The metabolic phenotype of DKO is largely similar to *CB1*$^{-/-}$ mice, suggesting that adiponectin is not a key mediator of the metabolic effects of *CB1*. Interestingly, *CB1*$^{-/-}$ mice showed reduced pro-inflammatory macrophage polarization in both peritoneal macrophages and adipose tissue macrophages compared to WT mice; in contrast, DKO mice exhibited increased pro-inflammatory macrophage polarization in these macrophages compared to *CB1*$^{-/-}$ mice, suggesting that adiponectin is an important mediator of the inflammatory effect of *CB1*.

CONCLUSION

Our findings reveal that *CB1* functions through both adiponectin-dependent and adiponectin-independent mechanisms: *CB1* regulates energy metabolism in an adiponectin-independent manner, and inflammation in an adiponectin-dependent manner. The differential effects of adiponectin on *CB1*-mediated metabolic and inflammatory functions should be taken into consideration in *CB1* antagonist utilization.

Key Words: Cannabinoid type 1 receptor; Adiponectin; Thermogenesis; Macrophages; Inflammation; Insulin resistance

Core Tip: Antagonists of cannabinoid type 1 receptor (*CB1*) have been shown to promote body weight loss and improve insulin sensitivity. Cannabinoids have been shown to regulate adiponectin. However, it is unclear whether adiponectin is a key mediator of the functions of *CB1*. We compared metabolic and inflammatory phenotypes of *CB1*-null *vs* *CB1*/adiponectin double-knockout mice. Our findings reveal that *CB1* functions through both adiponectin-dependent and adiponectin-independent mechanisms: *CB1* regulates energy metabolism in an adiponectin-independent manner, and inflammation in an adiponectin-dependent manner.

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INTRODUCTION

The incidence of obesity has increased rapidly during recent decades, particularly in developed/industrialized countries. Obesity increases the incidences of hyperinsulinemia, insulin resistance, type 2 diabetes, dyslipidemia, atherosclerosis, hypertension, inflammation, and cancer[1,2]. Endocannabinoids are key regulators of food intake and energy metabolism, and the effects are mediated through the activation of the cannabinoid type 1 receptor 1 (*CB1*)[3,4]. Recent studies have demonstrated that blocking the activity of the endogenous cannabinoid system might be a strategy for the treatment of obesity and metabolic syndrome[5-7].

Previous study demonstrated that *CB1* knockout mice consume less food and have reduced body weight[4,8]. Rimonabant, a specific antagonist of *CB1*, reduces food intake by blocking the orexigenic effect of cannabinoids[9]. There is also evidence that endogenous cannabinoids regulate energy expenditure[10]. It has been shown that

Institutional Animal Care and Use Committee at Baylor College of Medicine, Houston, TX, United States).

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virally-induced hypothalamic *CB1* knockout mice showed no change in food intake, but did show less body weight gain over time due to increased energy expenditure; and the mRNA expression of β3-adrenergic receptor and uncoupling protein-1 (UCP-1) was elevated in the brown adipose tissue (BAT)[10]. There are also data showing that rimonabant alleviates dyslipidemia and obesity *via* a BAT thermogenesis-mediated increase of energy expenditure[9]. It has been shown that peripheral *CB1* blockade is effective in activating thermogenesis in BAT to mitigate dyslipidemia and obesity[11], which suggests that the function of *CB1* in BAT can be peripherally mediated and is not necessarily dependent on its central action. These results suggest that endocannabinoids may regulate energy metabolism by binding to *CB1* expressing cells in peripheral white adipose tissue (WAT)[8] and/or BAT[9].

Adiponectin, an adipokine with insulin-sensitizing functions, has been reported to be relevant in many metabolic diseases such as obesity, and with associated complications such as diabetes, hyperinsulinemia, insulin resistance, dyslipidemia, hypertension, and inflammation[12]. Adiponectin treatment reduces body weight, improves hyperglycemia, ameliorates hyperinsulinemia and insulin resistance, and increases fatty acid oxidation and lipid clearance, in animal models of obesity and diabetes[13,14]. One of the most intriguing consequences of rimonabant treatment is increased adiponectin gene expression in adipose tissue of diet-induced obese (DIO) mice[9] and in cultured adipocytes[15]. However, the rimonabant-treated adiponectin- and leptin-deficient mice exhibit significantly ameliorated insulin resistance, which suggests that rimonabant reduces insulin resistance *via* both adiponectin-dependent and adiponectin-independent mechanisms[16]. These results suggest that rimonabant may regulate adiponectin expression in adipocytes, and the metabolic effects of rimonabant, at least in part, could be due to enhanced adiponectin secretion.

To determine whether adiponectin is indeed required for the peripheral functions of *CB1*, we used a genetic approach by breeding *CB1*$^{-/-}$ mice with adiponectin-deficient mice to generate a mouse model lacking both *CB1* and adiponectin, *aka* double KO (DKO). We studied metabolic regulation such as thermogenesis and insulin sensitivity in these mice. The link between inflammation and obesity is now increasingly recognized and inflammation is considered a culprit of insulin resistance. Thus, we also characterized macrophage polarization in peritoneal macrophages and adipose tissue macrophages to elucidate whether *CB1* acts through adiponectin to modulate CB-1 mediated inflammation.

MATERIALS AND METHODS

Animals

All procedures using animal experiments were approved by the Institution of Animal Care and Use Committee at Baylor College of Medicine. All mice used in this study were congenic male mice. All mice were on a pure C57/6J background. To generate mice lacking both *CB1* and adiponectin, *CB1*$^{-/-}$ mice and *adiponectin*$^{-/-}$ mice were bred to each other to create compound heterozygotes that were *CB1*+/-/*adiponectin*+/-. In the second cross, compound heterozygotes were further bred to each other to yield homozygous *CB1*$^{-/-}$/*adiponectin*$^{-/-}$ (*aka* double-knockout DKO mice); *CB1*$^{-/-}$ adiponectin+/+ mice (aka *CB1*$^{-/-}$), and *CB1*+/+ adiponectin+/+ (aka WT mice). Age-matched male WT, *CB1*$^{-/-}$ and DKO were used in the studies. There were three groups of mice used in the study: (WT) control group, *CB1*$^{-/-}$ group, DKO group. Animals were housed under controlled temperature and lighting (75 ± 1 ℉; 12 h light-dark cycle). The diet was from Harlan-Teklad (2920X) and the diet compositions are as follows: 16% of calories from fat, 60% from carbohydrates, and 24% from protein. All experiments were approved by the Animal Care Research Committee of the Baylor College of Medicine.

Metabolic characterizations

Magnetic Resonance Imaging analysis of body composition was also carried out using an EchoMRI Whole Body Composition Analyzer (Echo MRI®, United States). Metabolic parameters were obtained using an Oxymax open-circuit indirect calorimetry Comprehensive Lab Animal Monitoring System (CLAMS) from Columbus Instruments (Columbus, OH, United States). Energy expenditure (EE) was calculated as the product of the value of oxygen (3.815 + 1.232 × RQ) and the volume of O_2 consumed. Respiratory quotient (RQ) ratio of VCO_2/VO_2 was then calculated[17]. Energy expenditure was normalized to both body weight and lean mass. Locomotor activity was measured using infrared beams to count the number of beam breaks

during the recording period. The CLAMS data was the average of 3 d of data that were collected after 3 d of acclimation.

Insulin tolerance test and glucose tolerance test

The Insulin tolerance test (ITT) and glucose tolerance test (GTT) were carried out on WT, *CB1*$^{-/-}$ and DKO mice. For ITT, after being fasted for 6 h, glucose of mouse tail blood was measured using One Touch Ultra glucose meter (lifeScan, New Brunswick, NJ, United States). It can detect glucose concentrations from 20 to 600 mg/dl using an electrochemical biosensor technology based on glucose oxidase chemistry. Mice then received an *i.p.* injection of human insulin (Eli Lilly Indianapolis, IN, United States) at a dose of 1.0 U kg-1 of body weight. Tail blood glucose concentration was measured at 0, 30, 60, 90 and 120 min after *i.p.* insulin injection. The GTT was carried out after the mice were fasted for 18 h overnight. The mice received *i.p.* injection of glucose (Sigma-Aldrich, St. Louis, MO, United States) at a dose of 2.0 g kg-1 body weight. The tail blood glucose was measured at 0, 15, 30, 60 and 120 min after glucose injection, and blood was collected for ELISA insulin analysis at 0, 15, 30 and 120 min after glucose injection.

Flow cytometry analysis

Peritoneal macrophage and stromal vascular (SV) cells of epididymal adipose tissues were fractionated as described[18,19]. Briefly, to get peritoneal macrophage, 5 ml of cold phosphate buffer saline (PBS) was injected into mouse peritoneal cavities immediately after anesthesia. After shaking the mice for 2-3 min, peritoneal fluid was harvested and spun down for peritoneal macrophages at 500 g for 5 min at 4 °C. The stromal vascular cells were isolated from the equal mass of epididymal adipose tissues using the collagenase digestion method. For flow cytometry analysis, same quantity cells (1×10^6) were subsequently re-suspended and stained with appropriate antibodies (F4/80 and CD11c for M1 type macrophage, or F4/80 and CD206 for M2 type macrophage) as described in our previous study[20]. Antibody information used in flow cytometry analysis is as follows: PE anti-mouse F4/80 antigen (eBioscience, San Diego, CA), FITC anti-mouse CD11c antigen (BD Bioscience, San Jose, CA), purified CD16/CD32 antigen (BD Bioscience, San Jose, CA), and APC anti-mouse CD206 antigen (BD Bioscience, San Jose, CA). All data were collected using FACScan and analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Analysis of gene expression

BAT and WAT were snap-frozen in liquid nitrogen and stored at -80 ℃. Total RNA was extracted from frozen tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was subsequently treated with DNase (Ambion, Austin, TX). RNA quality was assessed on 1.5% agarose gel electrophoresis in the presence of formaldehyde, and RNA concentration was determined by NanoDrop. The cDNA was synthesized from 1g RNA using the Superscript III First-Strand Synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen). Quantitative real-time RT-PCR was performed on an ABI7900 using the SYBR Green PCR Master Mix or the Taqman gene expression Master Mix (Applied Biosystems, Carlsbad, CA, United States). After amplification, the PCR product was subjected to 2% agarose gel electrophoresis. 18S RNA and -actin were used as internal controls. The primer sequences of quantitative RT-PCR are listed in Table 1 below.

Data analysis

Data are expressed as means ± SEM. Two groups were compared by *t*-test. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 23.0 statistical software (IBM, Armonk, NY, United States).

RESULTS

CB1 ablation increases energy expenditure, reduces adiposity, and improves insulin sensitivity

The body weights of *CB1*$^{-/-}$ mice were significantly lower than WT littermates; the analysis of body composition showed a markedly decreased percentage of fat mass in *CB1*$^{-/-}$ mice compared to WT mice (Figure 1A). We then assessed food intake, locomotor activity, and energy expenditure using CLAMS. Our data showed there was a trend of reduction but no significant difference in total daily food intake by *CB1*$^{-/-}$

Table 1 The sequences of reverse transcription-polymerase chain reaction primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
UCP-1	GTGAAGGTCAGAATGCAAGC	AGGGCCCCCTTCATGAGGTC
PGC-1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
IR	CAAAAGCACAATCAGAGTGAGTATGAC	ACCACGTTGTGCAGGTAATCC
IRS1	GCCTGGAGTATTATGAGAACGAGAA	GGGGATCGAGCGTTTGG
PPARγ2	GCCTATGAGCACTTCACAAGAAATT	TGCGAGTGGTCTTCCATCAC
GLUT4	GCCTTGGGAACACTCAACCA	CACCTGGGCAACCAGAATG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
CD11C	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
CD206	TGATTACGAGCAGTGGAAGC	GTTCACCGTAAGCCCAATTT
TNFα	GAGAAAGTCAACCTCCTCTCTG	GAAGACTCCTCCCAGGTATATG
IL-1β	TGTTCTTTGAAGTTGACGGACCC	TCATCTCGGAGCCTGTAGTGC
IL-6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT

mice compared to WT mice (Figure 1B). To further determine whether there is a difference in locomotor activity, we analyzed spontaneous locomotor activity of these mice. Neither total daily locomotor activity nor the locomotor activity during light or dark cycles was altered (Figure 1C). We next calculated energy expenditure and found that energy expenditure of *CB1*$^{-/-}$ mice was higher compared to their WT counterparts when normalized to body weight but no difference when normalized to lean mass (Figure 1D). Together, the results indicate that while *CB1* ablation reduces body weight and fat deposition, it may be a due to a combination of changes in food intake and exergy expenditure.

Next, we assessed the glycemic phenotype. ITT showed that *CB1*$^{-/-}$ mice were more responsive to insulin challenge than WT mice (Figure 1E). During GTT, there was no difference in glucose clearance following a glucose load in WT and *CB1*$^{-/-}$ mice; but remarkably, the insulin levels of *CB1*$^{-/-}$ mice were significantly lower, indicative of increased insulin sensitivity (Figure 1F). These results indicate that *CB1*$^{-/-}$ mice have improved insulin sensitivity, which is in line with reduced body weight and body fat.

Collectively, these data suggest that *CB1* is an important regulator of energy homeostasis and insulin sensitivity.

Adiponectin has little impact on CB1-mediated overall metabolic profile

To determine whether the metabolic effects of *CB1* are mediated by adiponectin, we compared the metabolic phenotypes of *CB1*$^{-/-}$ and DKO mice. The body weights of *CB1*$^{-/-}$ mice were similar to their age-matched DKO (Figure 2A). There were also no differences in fat mass and lean mass between *CB1*$^{-/-}$ and DKO mice. Indirect calorimetry analysis showed similar total food intake (Figure 2B) and locomotor activity (Figure 2C) between DKO and *CB1*$^{-/-}$ mice. Interestingly, compared to *CB1*$^{-/-}$ mice, DKO mice had increased energy expenditure when corrected either by total body weight or by lean weight (Figure 2D).

Furthermore, there was no difference in insulin sensitivity assessment of ITT between *CB1*$^{-/-}$ and DKO mice (Figure 2E). We further assessed glucose response during GTT: No difference was detected in glucose response, but interestingly, the insulin of DKO was lower at 15 min but higher at 120 min as compared to that of *CB1*$^{-/-}$ mice (Figure 2F). These data suggest that DKO mice have mostly similar metabolic profile, insulin sensitivity and glycemic response as *CB1*$^{-/-}$ mice, despite there are some varied insulin responses to glucose. Taken together, the effects of *CB1* on metabolism are dominant; adiponectin is not essential in mediating the metabolic effects of *CB1*.

CB1 ablation activates thermogenesis in BAT

To determine the underlying mechanisms of the increased energy expenditure observed in *CB1*$^{-/-}$ mice, we subsequently analyzed BAT collected from the mice. *CB1*$^{-/-}$ mice showed a decreased ratio of BAT: Body weight as compared to WT mice (Figure 3A). Mitochondrial uncoupling protein 1 (UCP1) is the hallmark regulator of mitochondrial biogenesis and thermogenesis; when activated, UCP1 dissipates the

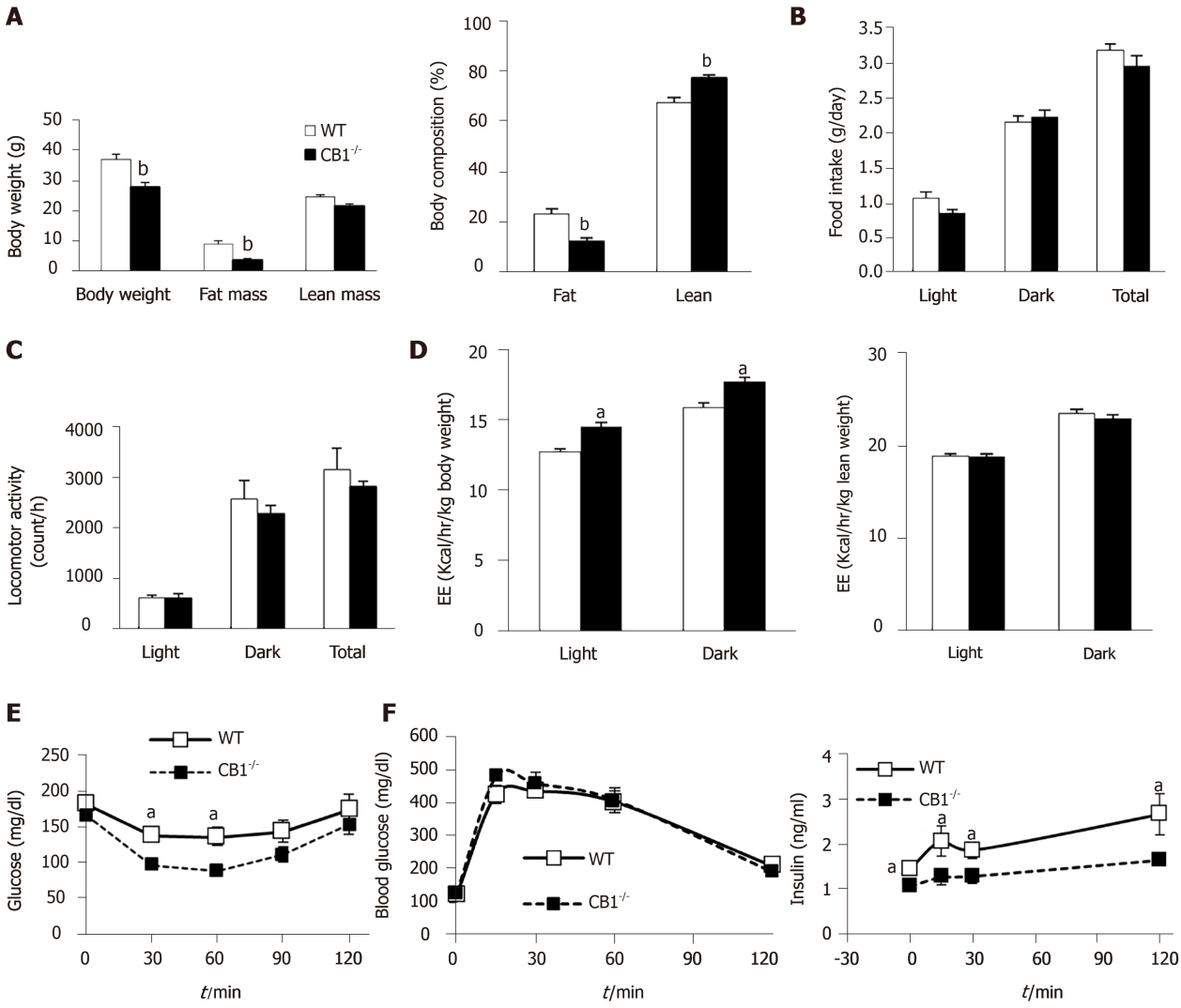

Figure 1 Cannabinoid type 1 receptor-null mice have reduced adiposity and improved insulin sensitivity. Wild-type (WT) and cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) male mice at 4 mo of age. A: Body weight and body composition; B: Daily food intake; C: Locomotor activity; D: Energy expenditure adjusted by body weight or lean mass; E: Insulin tolerance tests; F: Glucose tolerance tests at 5 mo of age. n = 5-7. $^{a}P < 0.05$, $^{b}P < 0.001$, WT *vs* *CB1*$^{-/-}$.

transmembrane proton gradient and generates heat[21]. UCP1 mRNA was increased in *CB1*$^{-/-}$mice as compared to WT controls (Figure 3B). Peroxisome proliferator-activated receptorγcoactivator-1 (PGC-1) is an upstream regulator of UCP1[22]. Indeed, PGC-1 expression was also increased in the *CB1*$^{-/-}$ mice when compared to that of WT mice (Figure 3B).

Our result in Figure 1 showed *CB1*$^{-/-}$ mice have higher insulin sensitivity compared to WT mice. Consistently, the gene expression of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) were increased in BAT of *CB1*$^{-/-}$ mice. Peroxisome proliferator-activated receptorsγ2 (PPARγ2) is an important master adipogenic regulator [11]. Here we found that PPARγ2 was higher in BAT of *CB1*$^{-/-}$ mice (Figure 3B). Glucose transporter type 4 (GLUT4) is a key mediator of glucose uptake in the adipose tissues[23]. As expected, GLUT4 expression in BAT of *CB1*$^{-/-}$ mice was increased (Figure 3B), supporting increased glucose uptake and consistent with increased heat production. Together, ablation of *CB1* increased BAT thermogenic activity, likely by modulating mitochondrial function, insulin signaling, adipogenesis, and glycose uptake signaling pathways in BAT.

We have reported that adiponectin has an important role in body temperature maintenance and thermogenesis. Here, we compared the weight of BAT depots in *CB1*$^{-/-}$ and DKO mice. There was also no difference in total weight or BAT percentage between *CB1*$^{-/-}$ and DKO mice (Figure 3C). The expression of thermogenic regulators UCP1 and PGC-1 was increased in BAT of DKO mice compared to that of *CB1*$^{-/-}$ mice, while the expression of IR and IRS-1, GLUT4, and PPARγ2 were unchanged (Figure 3D). These results suggest that while adiponectin may be an important mediator for the effect of *CB1* on mitochondrial genes in BAT, it is not critical for the

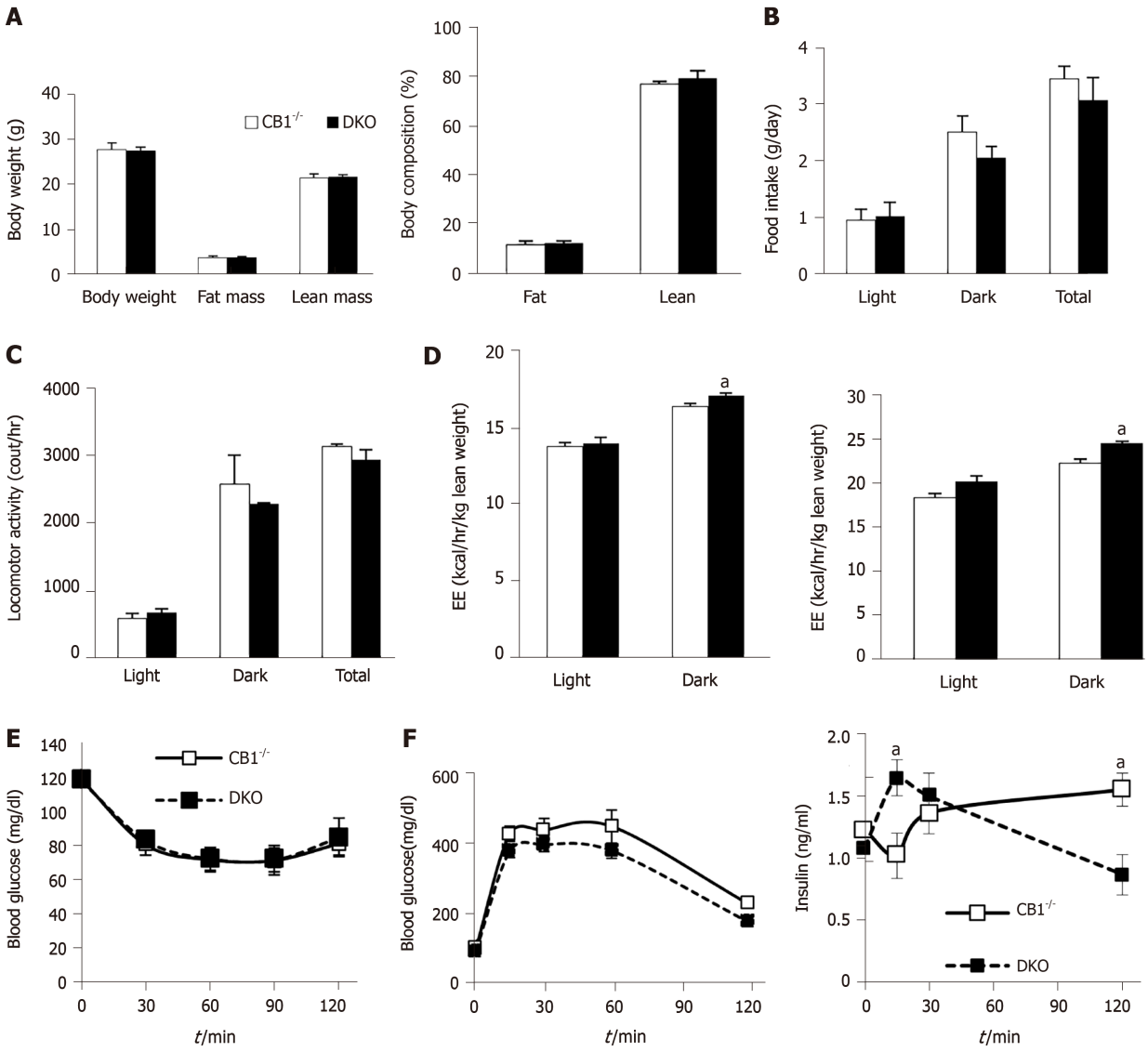

Figure 2 Cannabinoid type 1 receptor-null mice have similar adiposity and insulin sensitivity compared to double-knockout mice. Cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) and double-knockout (DKO) male mice at 4 mo of age. A: Body weight and body composition; B: Daily food intake; C: Locomotor activity; D: Energy expenditure adjusted by body weight or lean mass; E: Insulin tolerance tests; F: Glucose tolerance tests at 5 mo of age. n = 5-7. ^{a}P < 0.05, *CB1*$^{-/-}$ *vs* DKO.

regulation of *CB1* in insulin signaling, adipogenesis, and glucose uptake in BAT.

CB1 ablation promotes macrophage anti-inflammatory polarization

Macrophages have an important role in inflammation and insulin resistance[20]. To determine the underlying mechanisms of improved insulin sensitivity in *CB1*$^{-/-}$ mice, we conducted flow cytometry analysis on peritoneal macrophages and adipose tissue macrophages. M1-like macrophages are pro-inflammatory and M2-like macrophages are anti-inflammatory[20]. Peritoneal M1-like macrophages, as well as the M1/M2 ratio as a readout of inflammation, were significantly decreased in *CB1*$^{-/-}$mice compared to WT mice; this suggests that *CB1* ablation reduces systemic inflammation (Figure 4A). Since insulin resistance is closely linked to adipose tissue mass and adipose macrophages (ATM), we next assessed epididymal white adipose tissue. As expected, both the weight and the ratio of epididymal fat/body weight was lower in *CB1*$^{-/-}$ mice (Figure 4B). To assess the effect of *CB1* on ATM polarization, we isolated the stromal vascular fraction from epididymal adipose tissues. Our flow cytometry studies revealed that while M1 was slightly decreased, M2 was significantly increased in epididymal fat of *CB1*$^{-/-}$ mice (Figure 4C). The M1/M2 ratio of ATM was decreased in epididymal fat of *CB1*$^{-/-}$ mice (Figure 4C). Next, we studied the gene expression of macrophage markers of F4/80, CD11c, CD206, as well as proinflammatory cytokines of tumor necrosis factor-a (TNF-a), interelukin-1 (IL-1), and interelukin-6 (IL-6) in epididymal fat. The expression levels of F4/80, CD11c, CD206, TNF, IL-1 , and IL-6

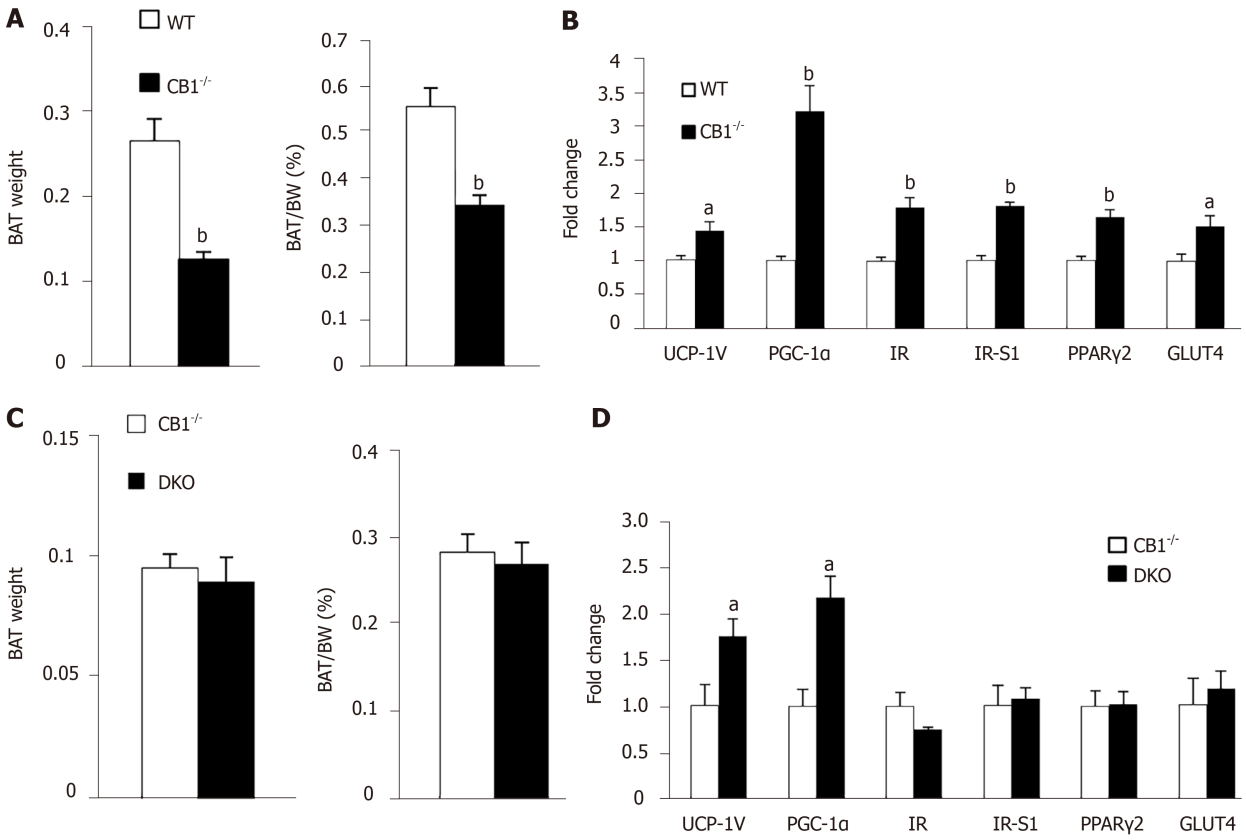

Figure 3 Double-knockout mice have similar expression of thermogenic genes compare to cannabinoid type 1 receptor-null mice. Wild-type (WT) and cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) male mice at 8 mo of age. A: Brown adipose tissue (BAT) weight and percentage of BAT depot; B: Quantitative real-time RT-PCR analysis of gene expression in BAT. Cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) and double-knockout (DKO) male mice at 8 mo of age. C: BAT weight and percentage of BAT depot; D: Quantitative real-time RT-PCR analysis of gene expression in BAT. n = 6-8. $^{a}P < 0.05$, $^{b}P < 0.001$, WT *vs* *CB1*$^{-/-}$ or *CB1*$^{-/-}$ *vs* DKO.

were significantly decreased in the epididymal fat of *CB1*$^{-/-}$ mice compared to WT mice (Figure 4D), which is in agreement with the reduced inflammation revealed by flow cytometry analysis of ATM.

Adiponectin ablation abolishes the anti-inflammatory effect of CB1 deficiency

Adipose tissue releases adiponectin, which plays an important role in the regulation of energy metabolism and inflammation[24]. Intriguingly, our flow cytometry analysis showed increased pro-inflammatory peritoneal M1 macrophages and increased ratio of M1/M2 in DKO mice; this suggests that the adiponectin deletion abolishes the anti-inflammatory effect of *CB1* knockout (Figure 5A). Subsequently, we analyzed epididymal white adipose tissues of *CB1*$^{-/-}$ and DKO mice. There was no difference in the percentage of fat depot: Body weight between *CB1*$^{-/-}$ and DKO mice (Figure 5B). Our flow cytometry studies further revealed that the M1/M2 ratio of ATM was increased in epididymal fat of DKO mice compared to *CB1*$^{-/-}$ mice (Figure 5C). To investigate the effect of adiponectin ablation of *CB1* on ATM-mediated inflammation, gene expressions of proinflammatory cytokines were evaluated in epididymal fat. The expression levels of F4/80, CD11c, CD206, TNF-a, IL-1, IL-6 and MCP-1 were significantly increased in epididymal fat of DKO mice as compared to *CB1*$^{-/-}$ mice (Figure 5D), in line with increased inflammation observed by flow cytometry.

Collectively, the data indicate that the *CB1* deficiency-induced anti-inflammatory effect on macrophage polarization is adiponectin-dependent, suggesting that adiponectin is a key mediator for the effect of *CB1* on inflammation.

DISCUSSION

The *CB1* blockade has been shown to ameliorate metabolic abnormalities of obese animals and to promote weight loss and improved insulin sensitivity[25]. Adipokine adiponectin is an insulin-sensitizer, and it has many beneficial effects that phenocopy

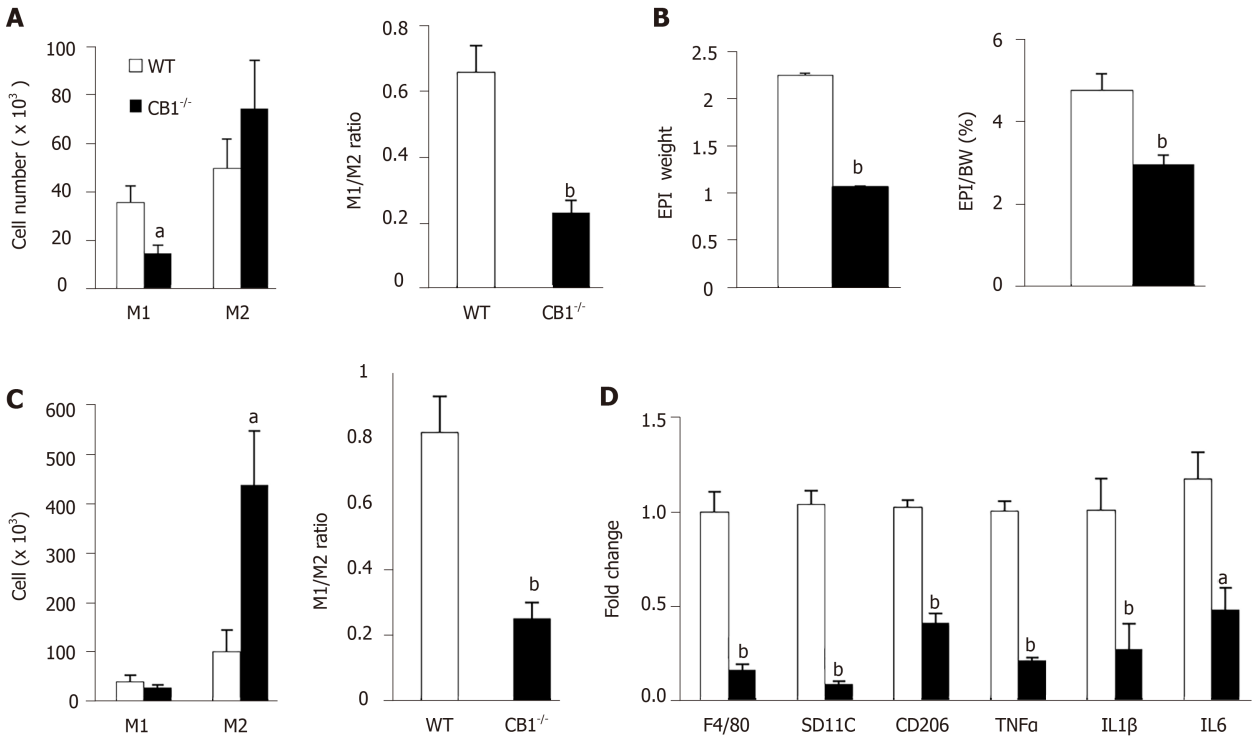

Figure 4 Cannabinoid type 1 receptor-null mice have reduced peritoneal and adipose tissue inflammation. Wild-type (WT) and cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) male mice at 8 mo of age. A: Flow cytometry analysis of M1 and M2 in peritoneal macrophages; B: Percentage of epididymal fat depot; C: Flow cytometry analysis of M1 and M2 in epididymal fat; D: Pro-inflammatory cytokines expression in epididymal fat. n = 6-8. $^{a}P < 0.05$, $^{b}P<0.001$, WT *vs* *CB1*$^{-/-}$ mice.

CB1 antagonists[12]. It has been shown that cannabinoids decrease adiponectin[26]. Moreover, *CB1* blocker rimonabant has been reported to increase the plasma adiponectin levels in obese and diabetic animal models[6,27,28]. Thus, adiponectin is thought to be a mediator of the effects of *CB1* antagonists such as rimonabant. However, the functional relationship between adiponectin and the endocannabinoid system is not fully defined. To determine whether *CB1* and adiponectin are functionally dependent on each other, we conducted a comparative study of the *CB1*$^{-/-}$ and DKO mice to investigate whether the adiponectin deletion abolishes the healthy phenotype of *CB1*$^{-/-}$ in metabolism and inflammation.

As expected in *CB1*$^{-/-}$ mice, we observed decreased body weight:fat mass, increased thermogenic activation in BAT, and improved whole-body insulin sensitivity. Interestingly, DKO mice showed changes similar to *CB1*$^{-/-}$ mice in the body weight:fat mass ratio, BAT thermogenic regulation, and insulin sensitivity. These results suggest that the beneficial metabolic effects of *CB1* blockage are not mediated by adiponectin. Our findings are mostly consistent with previous reports in literature, but with some differences which could be due to models of choice and/or diet variations. Watanabe *et al*[16] reported that rimonabant improved hepatic insulin resistance in both *ob/ob* and *adiponectin*$^{-/-}$*ob/ob* mice. Migrenne *et al*[29] reported that adiponectin is not required for body weight loss in diet-induced obese mice, but is required in rimonabant-induced improvement of insulin sensitivity. Our experiment was conducted with a genetic approach of loss-of-function with *CB1* knockout, not with *CB1* antagonist; under regular diet-feeding, not diet-induced obesity. It is possible that the impact of adiponectin on *CB1* metabolic regulation differs under different metabolic states. Indeed, Tam *et al*[30] reported a reversal of the HFD-induced hepatic steatosis and fibrosis by chronic administration of *CB1* blocker or adiponectin, but the reduction of adiposity and improved glycemic control are not affected by adiponectin, which is similar to our results.

The findings from our current study and others[4,8] support the idea of increased energy expenditure induced by *CB1* suppression, either by *CB1* blocker such as rimonabant or by *CB1* gene ablation. It is well known that BAT plays an important role in adaptive thermogenesis, and that thermogenic activation of BAT can directly affect metabolic rate through the function of mitochondrial protein UCP1. UCP1 is a key regulator of thermogenesis; it recruits free fatty acid into the mitochondrial matrix to dissipate as heat, depleting circulating lipids and increasing energy expenditure[31]. Previous studies demonstrated that rimonabant treatment increased the expression of

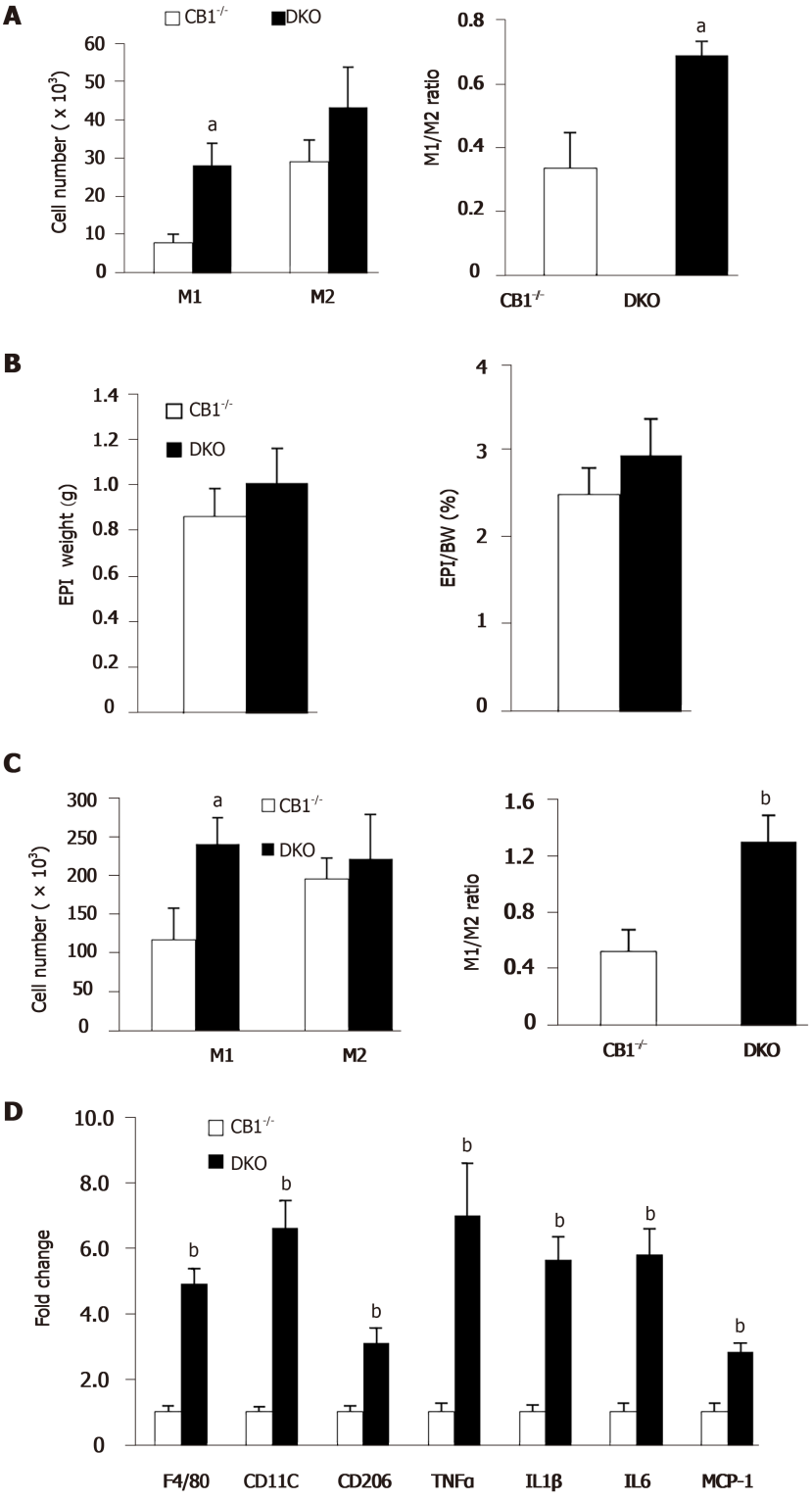

Figure 5 Double-knockout mice have increased peritoneal and adipose tissue inflammation compared to cannabinoid type 1 receptor-null mice. Cannabinoid type 1 receptor-null (*CB1*$^{-/-}$) and double-knockout (DKO) male mice at 7 mo of age. A: Flow cytometry analysis of M1 and M2 macrophages of peritoneal macrophages; B: Epididymal weight and percentage of epididymal depot; C: Flow cytometry analysis of M1 and M2 in stromal vascular fraction of epididymal fat; D: Pro-inflammatory cytokines expression of epididymal fat. n = 6-7. $^{a}P < 0.05$, $^{b}P < 0.001$, *CB1*$^{-/-}$ *vs* DKO mice.

UCP1 mRNA in BAT[32]. In metabolic profiling, DKO mice showed even higher energy expenditure than *CB1*$^{-/-}$ mice. Similarly, UCP-1 expression in BAT was higher in DKO mice than in *CB1*$^{-/-}$ mice. These results suggest that adiponectin deletion not diminishes the *CB1* deficiency-induced thermogenic activation in BAT. In the current study, we found that insulin signaling IR and IRS-1 gene expression in BAT was increased in *CB1*$^{-/-}$ mice, and the expression of these genes was no different between DKO and *CB1*$^{-/-}$ mice. Our thermogenic gene expression data in DKO showed that

adiponectin deletion further enhanced the thermogenic activation compared to *CB1*$^{-/-}$ mice, implying that the effect of *CB1* on thermogenesis is largely independent of adiponectin. The effect of adiponectin on thermogenesis is an area of ongoing debate currently. Qiao *et al*[33] reported that adiponectin suppresses thermogenic action in BAT to reduce energy expenditure. We reported that the core body temperature of adiponectin-null mice was not affected under normal housing temperature but reduced under cold temperature, supporting that adiponectin is required for maintaining body temperature in cold[24]. Different from our previous report, our current study was conducted under room temperature, so it is not surprising that the effect of adiponectin on thermogenic activation of *CB1*$^{-/-}$ mice is minimal.

Since metabolism and insulin sensitivity are closely linked to inflammation, we further studied the role of *CB1* deficiency in macrophages. Remarkably, both systemic (peritoneal macrophages) and tissue macrophages (ATM) showed an anti-inflammatory polarization shift, supporting reduced inflammation in *CB1*$^{-/-}$ mice. Especially, *CB1*$^{-/-}$ mice exhibited decreased pro-inflammatory M1 macrophages in peritoneal macrophages, less epididymal fat mass, and reduced M1/M2 ratio and pro-inflammatory cytokine expression in the epididymal fat as compared to WT mice. The results indicate that *CB1*$^{-/-}$ mice have reduced adiposity and adipose inflammation, which is consistent with improved systemic insulin sensitivity. Intriguingly, our study further revealed that DKO mice had an opposite profile of increased inflammation compared to *CB1*$^{-/-}$ mice, which suggested that adiponectin deletion reversed the anti-inflammatory effect of *CB1* deletion. The DKO mice exhibited an increase in pro-inflammatory M1 macrophages and M1/M2 ratio for both peritoneal macrophages and ATM, as well as elevated pro-inflammatory cytokine expression in epididymal fat compared to *CB1*$^{-/-}$ mice. The anti-inflammatory effect on *CB1*$^{-/-}$ mice was reversed in the DKO mice clearly demonstrates that adiponectin is required for the anti-inflammatory benefit of *CB1* antagonism, and the inflammation phenotype of *CB1* is adiponectin-dependent. These exciting results suggest that adiponectin counters the pro-inflammatory effect of cannabinoids, and the beneficial anti-inflammatory effect of *CB1* antagonists is dependent on adiponectin. Indeed, data from a mouse model of adipocyte-specific deletion of the *CB1* gene lends support to our conclusion[34]. Plasma adiponectin levels were significantly increased in the adipocyte-specific *CB1*-deleted mice, and adipocyte-specific deletion of *CB1* was shown to be sufficient to protect against diet-induced obesity and promote anti-inflammatory polarization towards alternatively-activated M2 macrophages.

CONCLUSION

In conclusion, our study demonstrates that *CB1* deletion activates thermogenesis and suppresses inflammation *via* adiponectin-independent and adiponectin-dependent pathways, respectively (Figure 6). Based on our findings, we conclude that there are differential pathways and mechanisms by which *CB1* utilizes to regulate metabolism and inflammation; that the effects on metabolism are adiponectin-independent and the effects on inflammation are adiponectin-dependent. *CB1* deletion increases plasma adiponectin[30,35], which promotes anti-inflammatory polarization of macrophages, thereby promoting the beneficial anti-inflammatory effect. Adiponectin is not required for *CB1*-mediated metabolism, but is required for *CB1*-mediated inflammation. A better understanding of the signaling crosstalk between *CB1* and adiponectin would facilitate further therapeutic development of *CB1* antagonists. Our study provides new insights to the comprehensive connection between *CB1* and adiponectin for regulation of energy homeostasis, insulin sensitivity and inflammation.

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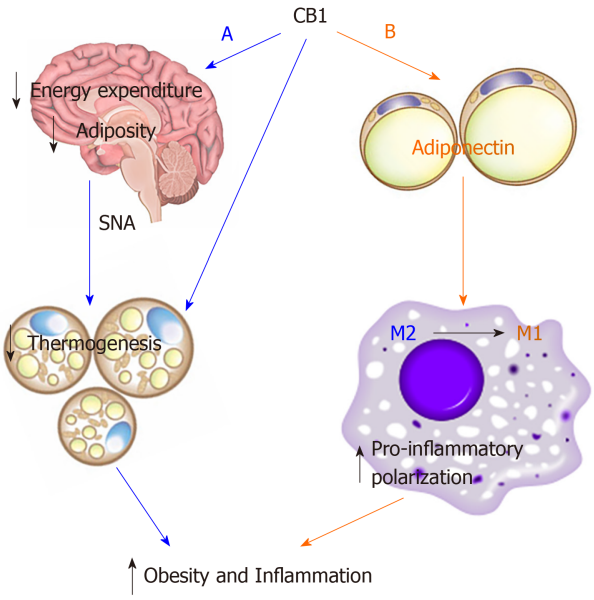

Figure 6 Schematic diagram of summary. Cannabinoid type 1 receptor (*CB1*) utilizes differential mechanisms in control of metabolism and inflammation. A: *CB1* decreases thermogenesis in BAT through sympathetic nerve activity to reduce energy expenditure and adiposity. So the effect of *CB1* on metabolism is adiponectin-independent; B: *CB1* suppresses adiponectin in adipose tissue, which diminishes the anti-inflammatory effect of adiponectin, thus promoting macrophage pro-inflammatory polarization. So the effect of *CB1* on inflammation is adiponectin-dependent. Thus, *CB1* utilizes differential mechanisms in control of metabolism and inflammation: its effect on metabolism is adiponectin-independent while effects on inflammation are adiponectin-dependent. Thus, adiponectin is not required for *CB1*-mediated metabolism, but is required for *CB1*-mediated inflammation.

ARTICLE HIGHLIGHTS

Research background

Antagonists of cannabinoid type 1 receptor (*CB1*) have been shown to promote body weight loss and improve insulin sensitivity.

Research motivation

Cannabinoids is implicated in regulation of adiponectin. However, the mediators of *CB1* actions are not fully defined, specifically in regard to adiponectin signaling *in vivo*.

Research objectives

To determine whether adiponectin is indeed required for the peripheral functions of *CB1*.

Research methods

We compared metabolic and inflammatory phenotypes of *CB1*-null ($CB1^{-/-}$) vs. *CB1*/Adiponectin double-knockout (DKO) mice. We investigated the insulin sensitivity using insulin tolerance test and glucose tolerance test, and inflammation using flow cytometry analysis of macrophages.

Research results

$CB1^{-/-}$ mice significantly reduced body weight and fat mass without change of total daily food intake and locomotor activity compared to wild-type (WT) mice. $CB1^{-/-}$ mice showed increased energy expenditure and improved insulin sensitivity compared to WT mice. DKO showed no difference in body weight, adiposity, or insulin sensitivity, only showed a modestly elevated thermogenesis in BAT compared to $CB1^{-/-}$ mice. $CB1^{-/-}$ mice showed reduced pro-inflammatory macrophage polarization in both peritoneal macrophages and adipose tissue macrophages compared to WT mice; in contrast, DKO mice exhibited elevated pro-inflammatory macrophage polarization in these macrophages compared to that of $CB1^{-/-}$ mice.

Research conclusions

Our findings reveal that *CB1* functions through both adiponectin-dependent and adiponectin-independent mechanisms: *CB1* regulates energy metabolism in an

adiponectin-independent manner, and inflammation in an adiponectin-dependent manner.

Research perspectives

Adiponectin is not required for *CB1*-mediated metabolism but is required for *CB1*-mediated inflammation. To fully understand the direct interactions and regulatory mechanisms between *CB1* and adiponectin, further dissemination in co-culture system to might be beneficial.

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ORIGINAL ARTICLE

Case Control Study

Diabetic kidney disease: Are the reported associations with single-nucleotide polymorphisms disease-specific?

Marek Saracyn, Bartłomiej Kisiel, Maria Franaszczyk, Dorota Brodowska-Kania, Wawrzyniec Żmudzki, Robert Małecki, Longin Niemczyk, Przemysław Dyrla, Grzegorz Kamiński, Rafał Płoski, Stanisław Niemczyk

ORCID number: Marek Saracyn 0000-0002-5800-0500; Bartłomiej Kisiel 0000-0001-6311-0475; Maria Franaszczyk 0000-0002-0719-1933; Dorota Brodowska-Kania 0000-0002-3328-1537; Wawrzyniec Żmudzki 0000-0002-1015-456X; Robert Małecki 0000-0003-3127-6395; Longin Niemczyk 0000-0001-9698-9930; Przemysław Dyrla 0000-0002-9546-5614; Grzegorz Kamiński 0000-0002-2357-0634; Rafał Płoski 0000-0001-6286-5526; Stanisław Niemczyk 0000-0001-8151-860X.

Author contributions: Saracyn M, Kisiel B, Płoski R and Niemczyk S designed the study; Saracyn M, Brodowska-Kania D, Żmudzki W, Niemczyk L and Małecki R collected clinical data and blood samples; Franaszczyk M and Saracyn M, isolated DNA; Franaszczyk M and Płoski R genotyped SNP's; Kisiel B performed statistical analysis; Saracyn M, Kisiel B, Dyrla P, Kamiński G, Płoski R and Niemczyk S interpreted clinical and genetical data; Saracyn M, Kisiel B and Brodowska-Kania D searched literature; SaracynM, Kisiel B, Płoski R and Niemczyk S prepared the manuscript; all authors contributed to final writing, proof-reading of the manuscript; all authors approved the final version of the paper.

Marek Saracyn, Dorota Brodowska-Kania, Wawrzyniec Żmudzki, Stanisław Niemczyk, Department of Internal Diseases, Nephrology and Dialysis, Military Institute of Medicine, Warsaw 04-141, Poland

Marek Saracyn, Dorota Brodowska-Kania, Grzegorz Kamiński, Department of Endocrinology and Isotope Therapy, Military Institute of Medicine, Warsaw 04-141, Poland

Bartłomiej Kisiel, Clinical Research Support Center, Military Institute of Medicine, Warsaw 04-141, Poland

Maria Franaszczyk, Department of Medical Biology, Molecular Biology Laboratory, Institute of Cardiology, Warsaw 04-628, Poland

Robert Małecki, Department of Nephrology, Międzyleski Specialist Hospital in Warsaw, Warsaw 04-749, Poland

Longin Niemczyk, Department of Nephrology, Dialysis and Internal Diseases, Warsaw Medical University, Warsaw 02-097, Poland

Przemysław Dyrla, Department of Gastroenterology, Military Institute of Medicine, Warsaw 04-141, Poland

Rafał Płoski, Department of Medical Genetics, Medical University of Warsaw, Warsaw 02-106, Poland

Corresponding author: Marek Saracyn, MD, PhD, Associate Professor, Department of Internal Diseases, Nephrology and Dialysis, Military Institute of Medicine, 128 Szaserów Street, Warsaw 04-141, Poland. msaracyn@interia.pl

Abstract

BACKGROUND

The genetic backgrounds of diabetic kidney disease (DKD) and end-stage kidney disease (ESKD) have not been fully elucidated.

AIM

To examine the individual and cumulative effects of single-nucleotide polymorphisms (SNPs) previously associated with DKD on the risk for ESKD of diabetic etiology and to determine if any associations observed were specific for

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Institutional review board statement: This study was approved by the Military Institute of Medicine Ethics Committee, Warsaw, Poland (approval No. 5/WIM/2010).

Informed consent statement: All involved patients gave their informed consent.

Conflict-of-interest statement: The authors declare that they have no competing financial interests. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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DKD.

METHODS

Fourteen SNPs were genotyped in hemodialyzed 136 patients with diabetic ESKD (DKD group) and 121 patients with non-diabetic ESKD (NDKD group). Patients were also re-classified on the basis of the primary cause of chronic kidney disease (CKD). The distribution of alleles was compared between diabetic and non-diabetic groups as well as between different sub-phenotypes. The weighted multilocus genetic risk score (GRS) was calculated to estimate the cumulative risk conferred by all SNPs. The GRS distribution was then compared between the DKD and NDKD groups as well as in the groups according to the primary cause of CKD.

RESULTS

One SNP (rs841853; SLC2A1) showed a nominal association with DKD (P = 0.048; P > 0.05 after Bonferroni correction). The GRS was higher in the DKD group (0.615 ± 0.260) than in the NDKD group (0.590 ± 0.253), but the difference was not significant (P = 0.46). The analysis of associations between GRS and individual factors did not show any significant correlation. However, the GRS was significantly higher in patients with glomerular disease than in those with tubulointerstitial disease (P = 0.014) and in those with a combined group (tubulointerstitial, vascular, and cystic and congenital disease) (P = 0.018).

CONCLUSION

Our results suggest that selected SNPs that were previously associated with DKD may not be specific for DKD and may confer risk for CKD of different etiology, particularly those affecting renal glomeruli.

Key Words: Diabetic kidney disease; Chronic kidney disease; End-stage kidney disease; Single-nucleotide polymorphism; Diabetes mellitus

Core Tip: The genetic background of diabetic kidney disease (DKD) and end-stage kidney disease (ESKD) has not been fully elucidated. This study on a large population of dialyzed patients shows that single-nucleotide polymorphisms (SNPs) previously described in diabetes mellitus type 2 patients with DKD are not associated with the risk for ESKD of a diabetic background. Instead, the analyzed SNPs seem to correlate with glomerular kidney disease. These findings suggest that chronic kidney disease of different etiologies but the same dominant location of the pathological processes may share a common genetic background.

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INTRODUCTION

A diabetes mellitus (DM) epidemic is underway; in 2019, approximately 463 million people worldwide were estimated to have DM, and half of those cases were considered undiagnosed. By 2045, the prevalence is expected to increase to 700 million cases[1-3]. In Poland, around 3.5 million people (9.1% of the total population) suffer from DM[4]. Roughly 30% of patients with type 1 DM (DM1) and 40% with type 2 DM (DM2) develop a serious complication of the small renal vessels: diabetic kidney disease (DKD)[5,6]. The occurrence of DKD considerably worsens the long-term prognosis — the risk of premature death in end-stage kidney disease (ESKD) patients

quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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requiring renal replacement therapy (RRT) is 18-fold greater than in the general population[7]. Understandably, DKD is the strongest single predictor of death in a diabetic patient[8]. It is estimated that the number of DKD-associated deaths in DM patients increased by 94% from 1990 to 2012[9]. The recognized risk factors for DKD are hyperglycemia, acute kidney injury, hypertension, and obesity; however, age, gender, race, and family history are also influential factors[10].

Both clinical and epidemiological studies on DKD show a familial association of the disease[11-13], suggesting that heredity plays an important role in its development. Investigators aiming to elucidate the genetic basis of DKD have used two different approaches: candidate gene studies (CGS) and genome-wide association studies (GWAS). During the last two decades, CGS have tested more than 150 loci for their potential relationship with the renal complications of DM[14-16] and have reported an association between several single-nucleotide polymorphisms (SNPs) and albuminuria, glomerular filtration rate (GFR), DKD, and ESKD in both DM1 and DM2 patients. The majority of the proteins encoded by these genes are components of nephron (glomerulus, epithelium, and ion channels), but some are related to the extracellular matrix, immune response, phagocytosis, and cell migration[17]. During the last decade, GWAS have replaced CGS as the study method of choice. The results of GWAS have confirmed some of the CGS findings and have also revealed new associations[18,19]. These initial findings must be corroborated in replication studies in different populations.

With the increasing number of identified genetic risk factors for multifactorial diseases, tools that assess the cumulative impact of these factors on disease risk have been developed. One such tool is the genetic risk score (GRS), which our group has successfully applied in earlier studies[20,21]. GRS is particularly useful in situations wherein the individual effect carried by a single genetic variant is small. Previous studies have shown the capability of GRS to measure the genetic risk of various multifactorial diseases, including myocardial infarction, atrial fibrillation, stroke, rheumatoid arthritis, and psoriasis[22]. Thus, several studies have used this approach to evaluate the risk of renal complications in DM[23-25].

Previous analyses have searched for associations between genetic markers and the risk of diabetic renal complications but may have overlooked a critical issue: whether these genetic markers are specific for DKD or are, rather, associated with the risk for chronic kidney disease (CKD), regardless of its explicit underlying cause.

The aim of our study was to examine the individual and cumulative effects of SNPs previously described in DM2 patients with DKD on the risk for ESKD of diabetic etiology in a population of hemodialyzed (HD) patients and to determine if any associations observed were specific for DKD.

MATERIALS AND METHODS

This study was approved by the Military Institute of Medicine Ethics Committee, Warsaw, Poland. Informed consent was obtained from each patient. All procedures were performed in accordance with the Helsinki Declaration of 1975, revised in 1983.

Patients and controls

From an initial group of 1246 ESKD/HD patients, 136 consecutive patients with DM2 and DKD as the primary cause of ESKD were included in this study; they formed the DKD group. The control group was composed of 121 age-matched ESKD/HD patients with non-DKD (NDKD); this was the NDKD group. The inclusion criteria were as follows: (1) ESKD treated by hemodialysis; and (2) Age ≥ 18 years. The exclusion criterion was the presence of malignancy. The patients were recruited from the Military Institute of Medicine in Warsaw, Poland, and the Mazovian Centers of Dialysis in the following cities in Poland: Radom, Ciechanów, Grodzisk Mazowiecki, Maków Mazowiecki, Sokołów Podlaski, Skierniewice, Warszawa Międzylesie, Wołomin, and Otwock. Patients were classified as DKD or NDKD on the basis of histopathological examinations and diagnoses made by two independent nephrologists in each of the Mazovian Dialysis Centers. Nineteen DKD patients and 11 NDKD patients were excluded from the study because of poor DNA quality or incomplete genotyping data; the final DKD and NDKD groups consisted of 117 and 110 patients, respectively. Nineteen patients in the NDKD group were classified by nephrologists as having kidney disease of complex pathogenesis (CP group). A structured questionnaire was used to collect data regarding age, gender, smoking habits, body mass index (BMI), history of kidney disease in the pre-ESKD/HD period,

presence and volume of diuresis, and coexistence of hypertension and current treatment. Then, the patients were re-classified on the basis of the 2012 Kidney Disease Improving Global Outcomes (KDIGO) guidelines and CKD classification system based on the primary cause of their kidney disease. These classifications included (1) glomerular disease (GD); (2) tubulointerstitial disease (TID); (3) vascular disease (VD); and (4) cystic and congenital disease (CCD). Patients with CP were considered separately[26].

SNP selection

SNPs previously associated with DKD in DM2 patients were selected for this study[27-38]. The inclusion criteria for SNP selection were as follows: (1) Association with DKD in the presence of DM2 confirmed in a GWAS, meta-analysis, or large-scale case-control study; (2) Odds ratio (OR) ≥ 1.2 (or ≤ 0.83); and (3) Minor allele frequency ≥ 0.1 in a Caucasian population (based on data from the HapMap CEU). The exclusion criteria were as follows: (1) Association limited to non-Caucasian populations; or (2) Lack of studies on Caucasian populations. On the basis of these criteria, 14 SNPs were selected for genotyping. However, five of the genotyped SNPs were excluded from the analysis because of a low genotyping success rate (< 80%) or Hardy-Weinberg equilibrium (HWE) deviation: rs9521445 (*MYO16/IRS2*), rs1801133 (*MTHFR*), rs2241766 (*ADIPOQ*), rs5186 (*AGTR1*), and rs4880 (*SOD2*). The complete list of the analyzed SNPs is shown in Supplementary Table 1.

Genotyping

The salting out method was used to isolate DNA form whole blood samples[39]. For SNP genotyping, a custom array was designed (Taqman® OpenArray® Genotyping Plate, Custom Format 16 QuantStudio™ 12 K Flex, Life Technologies, Carlsbad, CA, United States), and genotyping was performed following the manufacturer's protocols on a QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, United States).

Statistical analysis

The statistical methods used in this study were reviewed by Kisiel B of the Clinical Research Support Center, Military Institute of Medicine, Warsaw, Poland. The differences in allele distribution between cases and controls as well as HWE were evaluated using the PLINK v1.07 statistical software package[40]. The deviation from HWE was considered significant at $P < 0.05$. The rest of statistical analyses was undertaken with the use of Statistica 12 package (StatSoft Inc). A Bonferroni correction (P value × number of tested SNPs) was used to adjust for multiple comparisons. To evaluate the cumulative risk of multiple loci a GRS (computed by summing the products of the number of risk alleles and the natural logarithm of the OR for each SNP) was calculated. For the calculation of GRS we used ORs from previous studies (Supplementary Table 1). The GRSs of the DKD and NDKD groups were compared using Student's *t*-test, whereas the GRSs of GD, VD, TID, CCD, and CP groups were compared using ANOVA. Pearson's test was used to assess the correlations between different parameters.

RESULTS

The clinical and demographic data of the subjects are presented in Table 1. The DKD and NDKD groups differed significantly with respect to BMI ($P = 0.01$) and treatment with beta-blockers ($P = 0.015$).

SNP associations with DKD are detailed in Table 2. Only one SNP (rs841853) showed a nominal association with DKD ($P = 0.048$; $P > 0.05$ after Bonferroni correction).

To evaluate the cumulative risk of multiple loci, we calculated GRS; it was slightly higher in the DKD group (0.615 ± 0.260) than in the NDKD group (0.590 ± 0.253), but the difference was not significant ($P = 0.46$). The GRS difference remained not significant ($P = 0.34$) after the exclusion of 19 CP patients from the NDKD group: 0.615 ± 0.260 for the DKD group and 0.582 ± 0.244 for the NKD group. The analysis of associations between GRS and the individual factors of gender, diuresis, and rate of CKD progression in the DKD and NDKD groups showed a significant correlation of GRS with diuresis in the NDKD group (0.643 ± 0.22 for diuresis > 500 mL *vs* 0.535 ± 0.253 for diuresis < 500 mL, $P = 0.035$, Table 3).

Table 1 Study and control group characteristics

	DKD (*n* = 117)	NDKD (*n* = 110)	*P* value
Age (yr)	68.94 (7.90)	69.87 (10.86)	0.46
Male sex (%)	57 (48.72)	66 (60.00)	0.088
Time-to-dialysis (yr)	5.25 (5.16)	6.23 (6.09)	0.20
Rapid progression of CKD (TTD ≤ 3 mo) (%)	6 (5.13)	6 (5.45)	0.91
Fast progression of CKD (TTD ≤ 1 yr) (%)	29 (24.79)	21 (19.09)	0.30
Slow progression of CKD (TTD > 5 yr) (%)	37 (31.62)	43 (39.09)	0.24
BMI (kg/m^2)	30.21 (16.35)	26.01 (5.15)	**0.01**
Smoking ever (%)	52 (44.44)	58 (53.21)[1]	0.19
Preserved diuresis (%)	97 (82.91)	85 (77.27)	0.29
24 h diuresis > 500 mL (%)	49 (41.88)	46(41.82)	0.99
Hypertension (%)	114 (97.44)	102 (92.73)	0.099
ACE-I (%)	50 (43.48)[2]	39 (39.39)[4]	0.54
ARB (%)	17 (14.78)[2]	12 (11.76)[5]	0.51
Beta-blockers (%)	89 (76.72)[3]	91 (89.22)[5]	**0.015**
Statins (%)	76 (66.09)[2]	57 (58.16)[6]	0.23

[1]Data available for 109 patients.
[2]Data available for 115 patients.
[3]Data available for 116 patients.
[4]Data available for 99 patients.
[5]Data available for 102 patients.
[6]Data available for 98 patients.
Significant differences are highlighted in bold. ACE-I: Angiotensin-converting-enzyme inhibitors; ARB: Angiotensin II receptor blockers; BMI: Body mass index; CKD: Chronic kidney disease; DKD: Diabetic kidney disease; NDN: Non-diabetic kidney disease; TTD: Time-to-dialysis (time between the diagnosis of chronic kidney disease and start of hemodialysis).

Table 2 Single-nucleotide polymorphisms' associations with diabetic kidney disease

SNP	DKD		NDKD		OR	*P* value
	E	N	E	N		
rs1617640	123	111	119	101	0.94 (0.65-1.36)	0.78
rs841853	84	150	60	160	1.49 (1.00-2.23)	0.048
rs1800783	67	167	69	151	0.88 (0.59-1.31)	0.53
rs1531343	23	211	17	203	1.30 (0.68-2.51)	0.43
rs1800470	91	143	93	127	0.87 (0.60-1.26)	0.46
rs759853	94	140	74	146	1.32 (0.90-1.94)	0.15
rs1801282	40	194	35	185	1.06 (0.66-1.79)	0.73
rs13293564	104	130	91	129	1.13 (0.78-1.65)	0.51
rs2268388	29	205	32	188	0.83 (0.48-1.43)	0.50

DKD: Diabetic kidney disease; E: Effect allele; NDKD: Non-diabetic kidney disease; N: Non-effect allele; OR: Odds ratio; SNP: Single nucleotide polymorphism.

We analyzed the distribution of GRS in the GD, TID, VD, CCD, and CP groups using ANOVA; the differences between groups were not significant (Table 4). However, *post hoc* analysis showed a significantly higher GRS in the GD group (0.628 ± 0.256) than in the TID group (0.461 ± 0.218) (P = 0.014) as well as a higher GRS in the GD group (0.628 ± 0.256) than in the combined TID+VD+CCD group (0.536 ± 0.235) (P

Table 3 Associations between genetic risk score and different parameters in diabetic kidney disease and non-diabetic kidney disease patients

Parameter	DKD + NDKD, *n* = 227		DKD, *n* = 117		NDKD[1], *n* = 91	
	GRS, mean ± SD	*P* value	GRS, mean ± SD	*P* value	GRS, mean ± SD	*P* value
Rapid progression (TTD ≤ 3 mo *vs* > 3 mo)	0.602 ± 0.233 vs 0.603 ± 0.258	0.99	0.518 ± 0.273 *vs* 0.620 ± 0.260	0.34	0.687 ± 0.167 *vs* 0.574 ± 0.248	0.27
Fast progression (TTD ≤ 1 yr *vs* > 1 yr)	0.592 ± 0.246 *vs* 0.606 ± 0.260	0.75	0.600 ± 0.229 *vs* 0.620 ± 0.270	0.71	0.522 ± 0.253 *vs* 0.593 ± 0.242	0.30
Slow progression (TTD > 5 yr *vs* ≤ 5 yr)	0.596 ± 0.256 *vs* 0.607 ± 0.258	0.77	0.629 ± 0.286 *vs* 0.608 ± 0.249	0.69	0.583 ± 0.230 *vs* 0.580 ± 0.257	0.96
Diuresis (preserved diuresis *vs* no diuresis)	0.614 ± 0.260 *vs* 0.557 ± 0.239	0.18	0.622 ± 0.265 *vs* 0.578 ± 0.235	0.49	0.605 ± 0.244 *vs* 0.503 ± 0.235	0.09
24h diuresis > 500 mL (> 500 mL *vs* ≤ 500 mL)	0.630 ± 0.244 *vs* 0.583 ± 0.265	0.17	0.621 ± 0.258 *vs* 0.611 ± 0.263	0.83	0.643 ± 0.220 *vs* 0.535 ± 0.253	**0.035**
Male sex (males *vs* females)	0.639 ± 0.265 *vs* 0.591 ± 0.255	0.31	0.617 ± 0.263 *vs* 0.586 ± 0.249	0.37	0.589 ± 0.251 *vs* 0.568 ± 0.235	0.70

[1]Cases with complex pathogenesis excluded from analysis.

DKD: Diabetic kidney disease; NDKD: Non-diabetic kidney disease; GRS: Genetic risk score; TTD: Time-to-dialysis (time between the diagnosis of chronic kidney disease and start of hemodialysis). Significant differences are highlighted in bold.

Table 4 The distribution of genetic risk score in particular end-stage kidney disease subgroups

Group	GRS	*P* value	Post-hoc analysis	
Classification: (1) GD, (2) TID, (3) VD, (4) CCD, (5) CP				
		0.09	GD *vs* TID	***P* = 0.014**
			GD *vs* VD	*P* = 0.12
GD (*n* = 146)	0.628 ± 0.256		GD *vs* CCD	*P* = 0.61
TID (*n* = 16)	0.461 ± 0.218		GD *vs* CP	*P* = 0.97
VD (*n* = 33)	0.551 ± 0.269		TID *vs* VD	*P* = 0.24
CCD (*n* = 13)	0.590 ± 0.138		TID *vs* CCD	*P* = 0.18
CP (*n* = 19)	0.629 ± 0.298		TID *vs* CP	*P* = 0.051
			VD *vs* CCD	*P* = 0.64
			VD *vs* CP	*P* = 0.29
			CCD *vs* CP	*P* = 0.66
Classification: (1) GD, (2) TID+VD+CCD, (3) CP				
GD (*n* = 146)	0.628 ± 0.256	0.055	GD *vs* TID+VD+CCD	***P* = 0.018**
TID+VD+CCD (*n* = 62)	0.536 ± 0.235		GD *vs* CP	*P* = 0.97
CP (*n* = 19)	0.629 ± 0.298		TID+VD+CCD *vs* CP	*P* = 0.16
Classification: (1) GD, (2) TID+VD+CCD+CP				
GD	0.628 ± 0.256	0.051	N/A	
TID+VD+CCD+CP	0.558 ± 0.253			

Genetic risk score is presented as mean ± SD. GRS: Genetic risk score; GD: Glomerular disease; TID: Tubulointerstitial disease; VD: Vascular diseases; CCD: Cystic and congenital disease; CP: Complex pathogenesis; N/A: Not applicable.

= 0.018). The analysis of associations between GRS and the individual factors of gender, diuresis, and rate of CKD progression did not show any significant correlation in the GD group (Supplementary Table 2).

DISCUSSION

To the best of our knowledge, this study may be the first to assess the genetic risk of DKD and other nephropathies leading to ESKD and RRT in a relatively large group of HD patients. Unexpectedly, none of the selected SNPs previously described in DM2 patients and associated with DKD development significantly correlated with DKD in our group of ESKD patients requiring hemodialysis, except one (rs841853) that showed a nominal association with DKD (P = 0.048; P > 0.05 after Bonferroni correction). Moreover, the GRS composed of all genotyped SNPs, carrying the cumulative risk of all tested loci, did not differ significantly between the DKD and NDKD groups and did not associate with any of the analyzed clinical parameters, including the rate of renal failure progression. This observation may have three explanations: (1) The analyzed SNPs are not associated with DKD in a Polish population; (2) The analyzed SNPs are associated with earlier stages of DKD; or (3) The analyzed SNPs are not specific for DKD but are associated with different kidney diseases and their progression.

It should be emphasized that many earlier studies on the genetic background of DKD have used DM patients without DKD as controls. This approach has identified risk factors for DKD development but has been unable to assess their specificities. That is, the genetic factors that have been pinpointed to be associated with DKD are not necessarily specific for DKD. To address this issue, we re-classified our patients on the basis of the KDIGO guidelines and found that GRS was significantly higher in the GD group than in the TID group and the combined TID+VD+CCD group. This observation suggests that these SNPs that were previously described as associated with DKD are in fact associated with the risk of CKD and ESKD of different etiologies, particularly those primarily affecting renal glomeruli.

In recent years, research has focused on the genetic basis of DKD. Nearly 160 genes have been implicated in DKD development as found in CDS and GWAS, and there appears to be a multigenic etiology of disease as gauged by GRS[14-19]. In a study involving 1100 DM2 patients, Wang *et al*[23] demonstrated that a GRS composed of six SNPs in genes involved in lipid metabolism (*PON1, PON2, CETP*), hemostasis (*ITGA2*) , and inflammation (*LTA1, LTA3*) was associated with a significantly higher risk of DKD; risks were lower when SNPs were individually assessed. Todd *et al*[24] examined a GRS composed of 32 validated BMI loci to determine the relationship between BMI and macroalbuminuria, ESKD, and DKD and found that a 1 kg/m^2 higher BMI increased the risk of macroalbuminuria by 28%, of ESKD by 43%, and of DKD by 33%. Barbieux *et al*[25] explored the association between 18 CKD-related SNPs and CKD G5 in 1,300 DM2 patients and 300 DM1 patients; however, neither a single SNP nor the 18-SNP GRS was linked to the deterioration of renal function, need for RRT, or death. Our study cannot be directly compared with the ones referenced above because it followed a completely different, exclusively DKD-related set of SNPs and a dissimilar study design in which non-diabetic patients were used as controls for diabetic ESKD patients.

The main finding of our study is that SNPs reported to be associated with DKD alone may, in fact, be correlated with CKD of different etiology. In this context, two papers are worth mentioning. A large-scale GWAS on 130000 subjects of European ancestry by Pattaro *et al*[41] showed that 53 known and novel SNP loci were highly correlated with GFR in individuals with or without diabetes; the effects on both patient groups were of similar magnitude. A study of even larger scale (including over 280000 subjects from the Million Veteran Program, over 765000 subjects from the CKD Gen Consortium, and more than 90000 diabetic patients) published by Hellwege *et al* [42] made similar observations: 32 SNP loci (17 known and 15 new) were significantly and with similar effect size associated with GFR in both diabetic and non-diabetic groups. Of course, reduced GFR is not synonymous with CKD (or DKD) and ESKD; however, the findings by Pattaro *et al*[41] and Hellwege *et al*[42] raise the possibility that CKDs of different etiologies may, to some extent, share a common genetic basis.

To address this issue, we reviewed recent publications regarding the possible relationship of our SNPs with CKD/ESKD of etiology other than DKD[43-50]. Most reports, whether on small or large populations, testing single or many variants, did not include the exact SNPs selected for our study, although they may have involved the same genes. For example, Hellwege *et al*[42] found an association between GFR and *NOS3* (nitric oxide synthase type 3) (rs3918226), but it was a different variation, which showed only a moderate linkage disequilibrium with our *NOS3* SNP (rs1800783) (R^2 = 0.17)[42]. Similarly, our SNPs were not any of those used in a GWAS by Wuttke *et al* [43], the largest meta-analysis to date. Essential differences exist between our study and the other studies cited, and simple comparisons are not applicable. First, the aforementioned studies characterized associations between SNPs and GFR in the

general population or in specific subpopulations such as diabetic patients, whereas our study aimed to delineate relationships between the selected SNPs and ESKD. Undoubtedly, changes in GFR are not synonymous with ESKD. In fact, a GWAS by Lin *et al*[44] indicated that the majority of loci do not overlap between early stages of CKD and ESKD, suggesting that distinct genetic factors may dominate at different stages of CKD. Second, a GWAS detects only the strongest associations, as corrections for a number of analyses are routinely used, possibly neglecting more nuanced but still meaningful effects on outcome measures. Third, studies on the general population, which include patients with CKDs of different etiologies, may simply not pick up disease-specific associations due to a water-downed subject pool.

The association of the SNPs selected for our study with CKDs of different etiologies, and not only DKD, is supported by the fact that their corresponding loci carry information affecting the structure and function of nephrons or interstitial renal tissue, the disorders of which are not restricted to diabetic etiology. In fact, the most common causes of CKD, such as DKD, hypertensive nephropathy, atherosclerotic nephropathy, renal mass reduction, obstructive nephropathy, and glomerular diseases, share many common pathophysiological pathways[45]. The factor initiating kidney injury is clearly different, but the progression of renal damage may involve mutual mechanisms, such as the renin-angiotensin-aldosterone system; endothelial, podocyte, mesangial, and tubular cell activation; platelet activation; common cytokine activation pathways (transforming growth factor-β1); the spread of inflammation; and repair of damaged tissues (fibrosis). A shared pathophysiology is even more probable in diseases affecting the same region of the kidney — in this case, the renal glomeruli.

This study has several strengths. First, the study group included only patients with ESKD, which addresses the suggestion made by earlier research that different CKD stages may be driven by distinctive genetic factors. Second, non-diabetic ESKD patients comprised our control group; this allowed us to answer the question whether the SNPs previously associated with DKD are specific for that type of CKD; indeed, we demonstrated that the SNPs in question were not specific for ESKD of diabetic etiology.

We must also acknowledge the major limitation of this study: its size. The study group was relatively small for a genetic analysis. However, owing to our strict inclusion criteria, the initial population of approximately 1200 patients with ESKD requiring RRT from dialysis centers in Mazovia, Poland, which has a general population of about 5.5 million, was winnowed down to those patients with documented DKD as the primary cause of ESKD and an appropriate, non-DKD control group with ESKD of etiology other than diabetes. Due to relatively small study group our results need to be confirmed in studies on larger populations.

CONCLUSION

Our study may be the first to demonstrate that selected SNPs that were previously associated with DKD may not be specific for DKD and may confer genetic risk for CKDs of different etiologies, particularly those affecting renal glomeruli. Our results need to be confirmed in studies on larger populations.

ARTICLE HIGHLIGHTS

Research background

A diabetes mellitus (DM) epidemic is underway; 40% patients with type 2 DM (DM2) develop a serious complication, diabetic kidney disease (DKD), and the occurrence of DKD considerably worsens the long-term prognosis. However, the genetic backgrounds of DKD and end-stage kidney disease (ESKD) have not been fully elucidated.

Research motivation

Previous studies have searched for associations between genetic markers and the risk of diabetic renal complications but may have overlooked a critical issue: whether these genetic markers are specific for DKD or are, rather, associated with the risk for chronic kidney disease and ESKD itself, regardless of its explicit underlying cause.

Research objectives

The aim of our study was to examine the individual and cumulative effects (as a genetic risk score, GRS) of SNPs previously described in DM2 patients with DKD on the risk of diabetic ESKD in a population of hemodialyzed patients and to determine if any associations observed were specific for DKD.

Research methods

Fourteen SNPs were genotyped in 136 patients with diabetic ESKD (DKD group) and 121 patients with non-diabetic ESKD (NDKD group). Patients were also classified on the basis of the KDIGO guidelines and CKD classification system based on the primary cause of CKD, such as glomerular disease (GD), tubulointerstitial disease (TID), vascular disease (VD), and cystic and congenital disease (CCD). Patients with complex pathogenesis (CP) were considered separately. The distribution of alleles was compared between diabetic and non-diabetic groups as well as between different sub-phenotypes. The weighted multilocus GRS was calculated to estimate the cumulative risk conferred by all SNPs. The distribution of GRS was then compared between the DKD and NDKD groups as well as in the groups according to KDIGO guidelines.

Research results

One SNP (rs841853; SLC2A1) showed a nominal association with DKD ($P = 0.048$; $P > 0.05$ after Bonferroni correction). The GRS was higher in the DKD group (0.615 ± 0.260) than in the NDKD group (0.590 ± 0.253), but the difference was not significant ($P = 0.46$). The analysis of associations between GRS and the individual factors of gender, diuresis, and rate of CKD progression in either study group did not show any significant correlation. However, the GRS was significantly higher in the GD group than in the TID group ($P = 0.014$) and the combined TID+VD+CCD group ($P = 0.018$).

Research conclusions

Our study may be the first to demonstrate that selected SNPs that were previously associated with DKD may not be specific for DKD and may confer genetic risk for CKDs of different etiologies, particularly those affecting renal glomeruli.

Research perspectives

Further studies are needed to confirm a similar correlation of other SNPs previously associated with DKD with the development and etiology of ESKD.

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Retrospective Cohort Study

Utility of oral glucose tolerance test in predicting type 2 diabetes following gestational diabetes: Towards personalized care

Riad Abdel Latif Bayoumi, Amar Hassan Khamis, Muna A Tahlak, Taghrid F Elgergawi, Deemah K Harb, Komal S Hazari, Widad A Abdelkareem, Aya O Issa, Rakeeb Choudhury, Mohamed Hassanein, Jeyaseelan Lakshmanan, Fatheya Alawadi

ORCID number: Riad Abdel Latif Bayoumi 0000-0001-8177-8129; Amar Hassan Khamis 0000-0002-8518-3066; Muna A Tahlak 0000-0002-0328-4569; Taghrid F Elgergawi 0000-0002-5916-2229; Deemah K Harb 0000-0001-9837-1843; Komal S Hazari 0000-0002-1879-8669; Widad A Abdelkareem 0000-0002-4774-6788; Aya O Issa 0000-0002-7818-2102; Rakeeb Choudhury 0000-0003-4047-0106; Mohamed Hassanein 0000-0001-6598-6972; Jeyaseelan Lakshmanan 0000-0002-9090-3005; Fatheya Alawadi 0000-0001-8911-866X.

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Riad Abdel Latif Bayoumi, Aya O Issa, Rakeeb Choudhury, Department of Basic Medical Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai 123, United Arab Emirates

Amar Hassan Khamis, Department of Biostatistics, HBMDC, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai 123, United Arab Emirates

Muna A Tahlak, Taghrid F Elgergawi, Deemah K Harb, Komal S Hazari, Widad A Abdelkareem, Department of Obstetrics and Gynecology, Dubai Health Authority, Dubai 123, United Arab Emirates

Mohamed Hassanein, Fatheya Alawadi, Department of Endocrinology, Dubai Health Authority, Dubai 123, United Arab Emirates

Jeyaseelan Lakshmanan, Department of Biostatistics, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai 123, United Arab Emirates

Corresponding author: Riad Abdel Latif Bayoumi, MBBS, PhD, Professor, Department of Basic Medical Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Building 14, Dubai Healthcare City, Dubai 123, Dubai.
riad.bayoumi@mbru.ac.ae

Abstract

BACKGROUND

Women with gestational diabetes mellitus (GDM) are at a seven-fold higher risk of developing type 2 diabetes (T2D) within 7-10 years after childbirth, compared with those with normoglycemic pregnancy. Although raised fasting blood glucose (FBG) levels has been said to be the main significant predictor of postpartum progression to T2D, it is difficult to predict who among the women with GDM would develop T2D. Therefore, we conducted a cross-sectional retrospective study to examine the glycemic indices that can predict postnatal T2D in Emirati Arab women with a history of GDM.

AIM

To assess how oral glucose tolerance test (OGTT) can identify the distinct GDM pathophysiology and predict possible distinct postnatal T2D subtypes.

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METHODS

The glycemic status of a cohort of 4603 pregnant Emirati Arab women, who delivered in 2007 at both Latifa Women and Children Hospital and at Dubai Hospital, United Arab Emirates, was assessed retrospectively, using the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria. Of the total, 1231 women were followed up and assessed in 2016. The FBG and/or the 2-h blood glucose (2hrBG) levels after a 75-g glucose load were measured to assess the prevalence of GDM and T2D, according to the IADPSG and American Diabetes Association (ADA) criteria, respectively. The receiver operating characteristic curve for the OGTT was plotted and sensitivity, specificity, and predictive values of FBG and 2hrBG for T2D were determined.

RESULTS

Considering both FBG and 2hrBG levels, according to the IADPSG criteria, the prevalence of GDM in pregnant Emirati women in 2007 was 1057/4603 (23%), while the prevalence of pre-pregnancy T2D among them, based on ADA criteria, was 230/4603 (5%). In the subset of women (n = 1231) followed up in 2016, the prevalence of GDM in 2007 was 362/1231 (29.6%), while the prevalence of pre-pregnancy T2D was 36/1231 (2.9%). Of the 362 pregnant women with GDM in 2007, 96/362 (26.5%) developed T2D; 142/362 (39.2%) developed impaired fasting glucose; 29/362 (8.0%) developed impaired glucose tolerance, and the remaining 95/362 (26.2%) had normal glycemia in 2016. The prevalence of T2D, based on ADA criteria, stemmed from the prevalence of 36/1231 (2.9%) in 2007 to 141/1231 (11.5%), in 2016. The positive predictive value (PPV) for FBG suggests that if a woman tested positive for GDM in 2007, the probability of developing T2D in 2016 was approximately 24%. The opposite was observed when 2hrBG was used for diagnosis. The PPV value for 2hrBG suggests that if a woman was positive for GDM in 2007 then the probability of developing T2D in 2016 was only 3%.

CONCLUSION

FBG and 2hrBG could predict postpartum T2D, following antenatal GDM. However, each test reflects different pathophysiology and possible T2D subtype and could be matched with a relevant T2D prevention program.

Key Words: Type 2 diabetes; Type 2 diabetes subtypes; Oral glucose tolerance test; Diabetes; Gestational diabetes mellitus subtypes

Core Tip: The oral glucose tolerance test (OGTT) remains the gold standard for assessing the risk of postnatal diabetes in women with gestational diabetes mellitus (GDM). Both the fasting blood glucose and 2-h blood glucose tests could predict postpartum abnormal glycemic status following antenatal GDM. However, each test reflects a different pathophysiology and possible subtype of type 2 diabetes (T2D). If fasting serum insulin measurements are added to an OGTT, additional data generated could distinguish T2D pathophysiology and possible subtypes. Information obtained could be used to match the T2D subtype with relevant prevention programs such as frequent follow-ups, lifestyle modifications, and new treatment protocols.

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INTRODUCTION

Hyperglycemia in pregnancy is observed in women who are already diagnosed with diabetes and in those whose first experience of hyperglycemia was during pregnancy. The latter is defined as gestational diabetes mellitus (GDM), a transitory condition in which women develop hyperglycemia during pregnancy that returns to normal after delivery. Women with GDM are at a seven-fold higher risk of developing type 2 diabetes (T2D) within 7-10 years after childbirth, compared with those with normoglycemic pregnancy[1-3]. However, it is unclear which of the women with GDM develop T2D.

Over the past 40 years, many studies have investigated the risk factors involved in the development of T2D after an index pregnancy with GDM[4-8]. The main identified factors included family history of diabetes, high body mass index (BMI), elevated fasting blood glucose (FBG) and elevated 2-h blood glucose (2hrBG) levels. Increased FBG levels has stood out as a significant predictor of postpartum progression to T2D [9-10]. Systematic reviews[11,12] summarized and quantified the contribution of risk factors to T2D development in women with a history of GDM.

GDM and T2D share similar genetic backgrounds and pathophysiological mechanisms regarding their development. Both conditions result from two major dysfunctions: a drop in peripheral sensitivity to insulin and failure of the β-cells of the pancreas to secrete insulin[13-14]. GDM is considered a variant of diabetes secondary to the release of placental hormones[15]. Therefore, it could be assumed that pregnancy reveals an existing predisposition for T2D.

T2D is increasingly being recognized as a highly heterogeneous disease, with varying clinical presentations, progressions, responses to treatment, and types of complications[16-21]. Both the FBG and 2hrBG tests have been used in the process of subtyping T2D and in explaining the heterogeneity of the disease. In a non-pregnant adult, the impaired uptake of glucose under fasting conditions, as detected by the FBG test, is reflective of hepatic insulin resistance with normal muscle insulin sensitivity accompanied by a decrease in early-phase insulin secretion. In contrast, the impaired tolerance for glucose, as detected by the 2hrBG test, indicates peripheral muscle insulin resistance, with defects in both early and late insulin secretions[22-28]. Therefore, FBG and 2hrBG tests seem to predict pathophysiology trajectories for T2D in non-pregnant adults and has, therefore, been used in the subtyping of the disease [16-21].

The FBG and 2hrBG tests have also been used to explain the heterogeneity of GDM. Several studies[29-32] have suggested that GDM could be subtyped into three groups: (1) the GDM-sensitivity group with predominant peripheral resistance to the action of insulin, exhibiting high BMI and elevated levels of FBG and serum leptin; (2) the GDM-secretion group with defective insulin secretion and low BMI values, similar to those in the normal glucose tolerance (NGT) group; and (3) the GDM-mixed group, characterized by both insulin sensitivity and secretion defects. Women in both the GDM-sensitivity and GDM-mixed groups have elevated FBG levels, compared with those in the NGT group. The OGTT remains the gold standard (GS) for the diagnosis of adult T2D[33] and for screening GDM[5]. The two main parameters of the OGTT, the FBG and 2hrBG tests, indicative of different pathophysiologies of the disease, are being consistently used in the attempts to subtype both adult T2D and GDM.

The Dubai Health Authority (DHA) has adopted a protocol for antenatal care based on the universal screening for hyperglycemia, using an OGTT at 24-28 gestational weeks. Although raised FBG levels has been said to be the main significant predictor of postpartum progression to T2D, it is difficult to predict who among the women with GDM would develop T2D. Therefore, in this retrospective cohort study, we examined the glycemic indices that can predict postnatal T2D in Emirati Arab women with a history of GDM. Data were extracted from routine hospital investigations of antenatal and postnatal care of women who delivered in 2007 in Latifa Women and Children Hospital and in Dubai Hospital, and were successfully followed-up in 2016.

MATERIALS AND METHODS

Patients

The present study was conducted in 2 hospitals: The Latifa Women and Children Hospital and Dubai Hospital; the 2 main public hospitals of the DHA, United Arab Emirates. The Latifa Women and Children Hospital is a 400-bed tertiary and referral hospital for obstetrics and gynecology and children care. Dubai Hospital is a 625-bed

center for referral of all medical and surgical specialties including obstetric services. Both hospitals share the same electronic health information system (HIS) called Salama.

Routine clinical and laboratory data for 4603 Emirati women, who delivered at the Latifa Women and Children Hospital (n = 3121) and Dubai Hospital (n = 1482) between January 1 and December 31, 2007, were collected from the "Salama" HIS. Of those women, 1231 (27%) were successfully followed up in 2016, and their data were compared with that of 2007. All 1231 women were included in the analysis. Therefore, no sample size or power analysis was performed.

Methods

Blood glucose was enzymatically assayed in the laboratories at the Latifa Women and Children Hospital and Dubai Hospital, using hexokinase as reference on the Cobas 6000 Analyzer (Roche Diagnostics, Basel, Switzerland). The measuring range of this method is 0.11-41.6 mmol/L (2-750 mg/dL). The coefficients of variation of the method are 0.7% and 1.2% for low and high blood glucose levels, respectively.

The routine protocol for antenatal care at the Latifa Women and Children Hospital and Dubai Hospital included universal screening for hyperglycemia using an OGTT at 24-28 gestational weeks. The FBG level and/or the 2hrBG level after a 75-g glucose load were measured to assess the prevalence of GDM and T2D, according to the IADPSG[5] and the ADA[8], respectively. GDM is defined as an FBG level of 5.1-6.9 mmol/L (92-125 mg/dL), and/or a 2hrBG level of 8.5-11.0 mmol/L (153-199 mg/dL) on a 2-h 75-g OGTT. DM is defined as an FBG level ≥ 7.0 mmol/L (126 mg/dL) and/or a 2hrBG of ≥ 11.1 mmol/L (200 mg/dL).

The ADA criteria for the diagnosis of diabetes in non-pregnant adults[8] has been adopted by the DHA and employed in our analysis of oral glucose tolerance testing. The FBG level and/or 2hrBG after a 75-g glucose load were measured to assess the prevalence of T2D. Diabetes was defined by a level of FBG ≥ 7.0 mmol/L (126 mg/dL) or a 2hrBG level ≥ 11.1 mmol/L (200 mg/dL). Impaired fasting blood glucose (IFG) was defined by a level of 5.6-6.9 mmol/L (100-125 mg/dL), while impaired glucose tolerance (IGT) was defined by a 2hrBG level of 7.8-11.0 mmol/L (140-199 mg/dL).

The glycemic status of the cohort of women (n = 1231) who were previously tested in 2007 was assessed again in 2016. Of those, 872 underwent FBG test only, 118 postprandial 2hrBG test only, while the remaining 241 had a complete OGTT.

The routine glycemic status of both the 4603 Emirati women in 2007 and the 1231 women who were followed up in 2016, were obtained from the Salama HIS. The women suspected of diabetes were confirmed and followed-up in either Hospital. The prevalence of T2D in the cohort of Emirati women tested in 2007 and the incidence of T2D during the 9-year period (2007-2016) were numerically calculated.

Data analysis

Data were analyzed using SPSS software version 23 (IBM, Chicago, IL, United States). All continuous data were described as mean ± SD, while the categorical data were described as number and percentage.

According to the IADPSG criteria, a woman will be considered to have GDM, at any time in her reproductive life, if her blood glucose is within the cut-off values for GDM and does not reach the cut-off values for diabetes. The prevalence of GDM in 2007 was calculated as percentage of women with OGTT blood glucose levels within the cut-off values, stipulated by the IADPSG criteria (m), divided by the total number of women in the specified cohort (N): (m/N) * 100. The incidence of diabetes in 2016 was calculated as the annual average of the difference over a 9-year period. Results were expressed as incidence rate and incidence density rate.

Specificity and sensitivity of FBG and 2hrBG in predicting T2D

The open-source R-4.02 statistical software was used to plot the receiver operating characteristic (ROC) curve for the OGTT. The women were categorized as having GDM or normal glycemia based on their FBG and 2hrBG levels in 2007. The diagnosis of T2D in 2016 was considered the GS, using HbA1c levels. The diagnosis of T2D was confirmed by correlation of FBG and 2hrBG values with HbA1c levels (Pearson correlation at 0.798; $P \leq 0.01$). To find the best cut off values for the 2007 FBG level, the actual values were plotted against the GS results (T2D or normal). At each cut off value for the 2007 FBG level, the sensitivity and specificity were calculated by forming a 2 by 2 table with the GS results[34-36].

The best cut off values for FBG for predicting T2D from GDM were calculated using the Youden Index: [(sensitivity + specificity) - 1]. However, on testing the 2hrBG level

in 2016, only five women were classified as having T2D. Therefore, performing an analysis to find the best cut off value for 2hrBG was not feasible.

Ethical considerations

This study was part of a project exploring hyperglycemia in pregnancy, funded by Al Jalila Foundation, Dubai, United Arab Emirates, under Grant No. AJF2015, dated November 8, 2015. Ethical approval was granted by the Dubai Scientific Research Ethics Committee of the DHA, with Reference No. DSREC: 12/2015_05; dated November 29, 2015. Data were anonymously collected for each participant in the study.

RESULTS

Demographics of Emirati women successfully followed up in 2016

Table 1 summarizes the age, BMI, parity, and outcomes of pregnancy in 1231 Emirati women, who delivered at Latifa Women and Children Hospital and Dubai Hospital in 2007 and were successfully followed up in 2016.

Prevalence of GDM and T2D in 2007

Combining the FBG and 2hrBG IADPSG criteria, the prevalence of GDM in pregnant Emirati women in 2007 was 1057/4603 (23%), while that of pre-pregnancy T2D, based on ADA criteria, was 230/4603 (5%). Among the subset of women (n = 1231) followed up in 2016 (Table 2), the prevalence of GDM in 2007 was 362/1231 (29.4%), while that of pre-pregnancy T2D was 36/1231 (2.9%). The proportion of women diagnosed with GDM based on a raised FBG level (267) was 1.8 times higher than that of those diagnosed based on a raised 2hrBG level (147).

Incidence of T2D in 2016

The glycemic status in 2016 of the same cohort of women (n = 1231), who were previously tested in 2007, is displayed in Table 3. Of those, 872 underwent FBG test only, 118 postprandial 2hrBG test only, while the remaining 241 underwent a complete OGTT. Based on the ADA criteria, the overall number of women who developed T2D increased from 36 (2.9%) in 2007 to 141 (11.5%) in 2016, a four-fold increase (Tables 2 and 3). The incidence of T2D over a 9-year period was estimated as follows: (141 – 36 = 105)/9 = 11.7 per 1000 Emirati women per year. All the women tested in the initial observation period in 2007 were also tested during the follow-up period in 2016. Therefore, the incidence density of T2D was the same as the incidence rate.

Conversion of GDM to T2D

To measure the conversion rate of GDM to T2D, the IADPSG glycemic indices of the cohort of Emirati women in 2007 (n = 1231) were cross tabulated against the ADA glycemic indices of the same cohort in 2016 (Table 4). Based on the isolated FBG, out of the 267 pregnant women with GDM in 2007, 69 (26 %) developed T2D, 89 (33%) developed IFG, 9 (3%) developed IGT, and the remaining 100 (38%) had normal glycemia in 2016.

Regarding isolated 2hrBG, out of the 147 pregnant women with GDM in 2007, 27 (18%) developed T2D, 53 (36%) developed IFG, 20 (14%) developed IGT, and the remaining 47 (32%) had normal glycemia in 2016. Based on associated FBG and 2hrBG, out of the 362 pregnant women with GDM in 2007, 96 (27%) developed T2D, 142 (39%) developed IFG, 29 (8.0%) developed IGT, and the remaining 95 (26%) had normal glycemia in 2016.

The conversion rate of GDM to IFG (33%-39%), was much higher than that of GDM to IGT (3%-14%). The prevalence of T2D, based on ADA criteria increased from 36/1231 (2.9%) in 2007 to 141/1231 (11.5%), in 2016. Women with raised FBG levels had a higher risk of developing T2D, compared with those with raised postprandial 2hrBG levels.

The sensitivity and specificity of FBG and 2hrBG tests in predicting T2D following GDM in Emirati women are shown in Table 5. The sensitivity of FBG was 82.3% (95%CI: 72.1, 90.0) while specificity was 55.1% (95%CI: 0.50, 0.60). The PPV for FBG of 24.3% suggests that, if a woman was positive for GDM in 2007, the probability of developing T2D in 2016 was about 24%. The negative predictive value (NPV) for FBG implied that, if a woman was negative for GDM in 2007, the probability of maintaining normal FBG levels was about 95%.

Table 1 Demographic characteristics of Emirati women who delivered in 2007 in Latifa Women and Children Hospital and Dubai Hospital, and were successfully followed up in 2016[1]

	mean ± SD
Number (*n*)	1231
Age (yr)	38.7 ± 6.1
BMI (kg/m^2)	31.1 ± 6.7
Parity (*n*)	5.74 ± 3.3
Live born (*n*)	4.53 ± 3.0
Still birth (*n*)	0.1 ± 0.3
Miscarriage (*n*)	1.1 ± 1.7

[1]Data were obtained in 2016.
BMI: Body mass index.

Table 2 Results of oral glucose tolerance test of 1231 Emirati pregnant women performed during their 24-28 wk of pregnancy in 2007, *n* (%)

Positive Diagnostic criteria[1]	Normal	GDM	T2D
Isolated FBG	995 (80.8)	215 (17.5)	21 (1.7)
Isolated 2hrBG	1121 (91.0)	95 (7.7)	15 (1.2)
Associated FBG and 2hrGB	1179 (95.8)	52 (4.2)	0 (0)
Total	833 (67.7)	362 (29.4)	36 (2.9)

[1]International Association of Diabetes and Pregnancy Study Groups (IADPSG)[5] criteria.
OGTT: Oral glucose tolerance test; GDM: Gestational diabetes mellitus; T2D: Type 2 diabetes; FBG: Fasting blood glucose; 2hrBG: 2-h blood glucose.

Table 3 Glycemic status of 1231 Emirati pregnant women, who were tested previously in 2007, and underwent post-natal glycemic tests in 2016

Diagnostic criteria[1]	Total number tested	Normal	Impaired FBG	IGT	Impaired FBG and IGT	T2D
FBG	872	542	203	-	-	127
Post-prandial 2hrBG	118	86	-	28	-	4
OGTT	241	146	23	52	10	10
Total	1231	774	226	80	10	141

[1]American Diabetes Association criteria for diagnosis of diabetes mellitus[8].
FBG: Fasting blood glucose; IGT: Impaired glucose tolerance; T2D: Type 2 diabetes; 2hrBG: 2-h blood glucose; OGTT: Oral glucose tolerance test.

The opposite is being observed in the predictability of T2D in 2016 using the 2hrBG in the diagnosis of GDM in 2007. The sensitivity of the 2hrBG test was 20.0% (95%CI: 0.05, 0.716), while the specificity was 88.3% (95%CI: 0.845, 0.92). The PPV value suggests that if a woman was positive for GDM in 2007, the probability of developing T2D in 2016 was about 3%. The NPV implied that if a woman was negative for GDM in 2007, the probability of maintaining normal 2hrBG levels was about 98%.

The sensitivities and specificities of various cut-off values for FBG in 2007 were estimated against the test results in 2016 (GS: T2D and normal). Using the Youden Index, a cut off value of FBG ≥ 103 mg/dL in 2007, above which T2D was diagnosed in 2016, was identified. This cut off value provided a sensitivity and specificity of 76.9% and 68.1% respectively. The area under the ROC Curve was 77.2% ($P < 0.001$). Thus, the FBG level ≥ 103 mg/dL at 2007 significantly predicted the T2D status in 2016. The 2hrBG levels could not be tested due to the small number who converted to T2D.

Table 4 Cross-tabulation of the glycemic status of Emirati women (*n* = 1231) who delivered in 2007, against their glycemic status in 2016, *n* (%)

IADPSG diagnostic criteria for GDM (2007)	ADA diagnostic criteria (2016)							
	Fasting				2 h			
	Total	Normal	IFG	T2D	Total	Normal	IGT	T2D
Fasting								
Normal	262 (100)	205 (78)	43 (16)	14 (5)	89 (100)	54 (61)	30 (34)	5 (5)
GDM	267 (100)	113 (42)	89 (33)	65 (25)	44 (100)	31 (70)	9 (21)	4 (9)
T2D	26 (100)	2 (8)	3 (11)	21 (81)	2 (100)	1 (50)	1 (50)	0 (0)
Total	555 (100)	320 (58)	135 (24)	100 (18)	135 (18)	86 (64)	40 (29)	9 (7)
2Hr								
Normal	670 (100)	495 (74)	138 (21)	37 (5)	252 (100)	197 (78)	51 (20)	4 (2)
GDM	147 (100)	68 (46)	53 (36)	26 (18)	34 (100)	13 (38)	20 (59)	1 (3)
T2D	26 (100)	7 (27)	5 (19)	14 (54)	4 (100)	1 (25)	2 (50)	1 (25)
Total	843 (100)	843 (68)	196 (23)	77 (9)	290 (100)	211 (73)	73 (25)	6 (2)

GDM: Gestational diabetes mellitus; T2D: Type 2 diabetes; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; FBG: Fasting blood glucose; 2hrBG: 2-h blood glucose.

Table 5 Sensitivity and specificity of fasting blood glucose and 2-h blood glucose in predicting type 2 diabetes following gestational diabetes mellitus in Emirati women[1]

IADPSG diagnostic criteria for GDM (2007)	ADA diagnostic criteria for T2D (2016)			
	FBG		2hrBG	
	T2D (*n*)	Normal (*n*)	T2D (*n*)	Normal (*n*)
GDM	65	202	1	33
Normal	14	248	4	248
Point estimates and 95%CIs				
True prevalence	0.149 (0.12, 0.183)		0.017 (0.006, 0.04)	
Sensitivity	0.823 (0.721, 0.9)		0.200 (0.005 0.716)	
Specificity	0.551 (0.504, 0.598)		0.883 (0.839, 0.918)	
Positive predictive value	0.243 (0.193, 0.299)		0.029 (0.001, 0.153)	
Negative predictive value	0.947 (0.912, 0.97)		0.984 (0.96, 0.996)	
Positive likelihood ratio	1.833 (1.586, 2.118)		1.703 (0.287, 10.12)	
Negative likelihood ratio	0.322 (0.198, 0.521)		0.906 (0.584, 1.408)	
Odds ratio	5.7 (3.108, 10.455)		1.879 (0.204, 17.32)	

[1]The R 4.02 statistical software was used to plot the receiver operating characteristic curve.
GDM: Gestational diabetes mellitus; T2D: Type 2 diabetes; FBG: Fasting blood glucose; 2hrBG: 2-h blood glucose.

DISCUSSION

Most studies that assessed the risk of developing T2D following a history of GDM were conducted in prospective clinical trials[1-7]. In contrast, our cross-sectional retrospective study analyzed the clinical and laboratory data of a cohort of Emirati Arab women, obtained from the routine clinical practice in a tertiary obstetrics set-up. The data were intended for clinical service; however, it proved to be useful for determining the risk of T2D in women with a history of GDM nine years earlier. The results suggested that both raised FBG and 2hrBG levels are sensitive glycemic

indicators of transition to prediabetes and T2D. Out of the 362 pregnant women with GDM in 2007, 27% developed T2D, 39% developed IFG, 8.0% developed IGT, and the remaining 26% had normal glycemia in 2016. The prevalence of T2D, based on the ADA criteria, increased from 2.9% in 2007 to 11.5%, in 2016. The conversion rate of GDM to T2D was higher in women with raised FBG levels (26%) than in women with raised 2hrBG levels (18%), indicating that the former group had a higher risk of developing T2D than the latter group. This was further supported by the OGTT ROC Curve indices. The PPV for FBG suggests that if a woman tested positive for GDM in 2007, the probability of developing T2D in 2016 was approximately 24%. The opposite is being observed in the predictability of T2D in 2016, using the 2hrBG. The PPV value suggests that, if a woman was positive for GDM in 2007, the probability of developing T2D in 2016 was only 3%. A similar trend of higher conversion rate of GDM to IFG was observed among women with raised FBG levels, compared with the rate of conversion of GDM to IGT among those with increased IGT. Our results agree with those of numerous previous studies referenced in several systematic reviews and meta-analyses[1-4,6-7,11-12].

The impaired uptake of glucose under fasting conditions, as detected by the FBG test, is reflective of hepatic insulin resistance with normal muscle insulin sensitivity accompanied by a decrease in the early-phase insulin secretion. In contrast, the impaired tolerance for glucose, as detected by the 2hrBG test, indicates peripheral muscle insulin resistance with defects in both early and late insulin secretion[22-28]. It is suggested that women with these two distinct metabolic states represent two distinct subtypes of GDM[29-32], depending on the defects in insulin sensitivity and/or secretion. A GDM-sensitivity group with predominant peripheral resistance to the action of insulin exhibited high BMI and elevated levels of FBG and serum leptin. Patients with defective insulin secretion, the GDM-secretion group, had low BMI values, similar to those in the NGT group. The third group is the GDM-mixed group, characterized by both insulin sensitivity and secretion defects. Women in both the GDM-sensitivity group and GDM-mixed group had elevated FBG levels compared with those in the NGT group. Earlier studies on the risk of T2D following GDM did not consider these proposed subtypes of GDM[1-3]. It is possible, therefore, that the GDM subgroup in our cohort of Emirati women, with raised FBG levels and higher conversion rate to T2D, is congruent with the GDM-sensitivity group characterized by peripheral insulin resistance; whereas, the GDM subgroup with raised 2hrBG levels reflected insulin secretion defects[29-32].

Subtyping of both adult non-pregnant with T2D[16-21] and GDM[29-32] patients represent serious attempts at resolving the heterogeneity of T2D, bringing the idea of personalized care closer, as pathophysiology is used to distinguish subtypes from each other. Different clinical management schemes are then tailored for each subtype. The use of the OGTT as a diagnostic tool has been discouraged over the past 20 years for various reasons[33]. However, insulin secretion and resistance could easily be deduced from assessing HOMA-B and HOMA-IR, if fasting serum insulin is measured during routine OGTT. The latter could then be instrumental in predicting T2D pathophysiology and possible subtypes.

A modified OGTT could become a powerful tool if extra parameters like fasting insulin and C-peptide, are measured simultaneously. It will help in identifying T2D subtypes and brings personalized patient care closer. Subtypes could then be matched with specific prevention programs like frequent follow-ups, lifestyle modifications, and new treatment protocols.

Limits of the study

This study, being retrospective in design, is limited. Fasting insulin and other hormones levels were not measured during routine hospital investigations. We could not obtain the indices for both secretion and resistance to the action of insulin, such as HOMA-B and HOMA-IR. A detailed prospective study will be essential for examining the trajectory of the conversion of GDM to T2D and the role that a modified OGTT could play in the dissection of the pathogenesis of the disease.

CONCLUSION

This cross-sectional retrospective cohort study, conducted among Emirati Arab women with GDM, revealed that raised antenatal FBG and 2hrBG levels could predict postpartum T2D; however, it suggested that each parameter may indicate a distinct T2D pathophysiology. Women with predominant peripheral resistance to the action of

insulin, who have raised FBG levels during pregnancy, were at a greater risk of developing T2D, compared with those with raised postprandial 2hrBG levels. It is suggested that, for the former group of women, postnatal management like frequent follow-ups, lifestyle modifications, and specific treatment protocols, should be applied to slow down the development of T2D and improve the quality of life for them and their newborns.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) is a common metabolic disorder of pregnancy. It has short- and long-term maternal, fetal, and neonatal complications. Women with GDM are at a seven-fold higher risk of developing type 2 diabetes (T2D) within 7-10 years after childbirth, compared with those with normoglycemic pregnancy.

Research motivation

There is emerging evidence that both GDM and T2D can be subtyped according to their pathophysiology. We attempted to examine the link between subtypes of GDM and the prediction of postnatal T2D.

Research objectives

To assess the utility of oral glucose tolerance test (OGTT) in the identification of distinct GDM pathophysiology and in the prediction of possible distinct postnatal T2D subtypes.

Research methods

The glycemic status of a cohort of 4603 pregnant Emirati Arab women, who delivered in 2007 in Dubai United Arab Emirates, was assessed retrospectively, using OGTT according to the International Association of Diabetes and Pregnancy Study Groups criteria. Of the total, 1231 women were followed up and assessed in 2016. The receiver operating characteristic curve for the OGTT was plotted and sensitivity, specificity, and predictive values of fasting blood glucose (FBG) and 2hrBG for T2D were estimated.

Research results

The prevalence of GDM in pregnant Emirati women in 2007 was 1057/4603 (23%), while the prevalence of pre-pregnancy T2D based on ADA criteria, was 230/4603 (5%). In the subset of women (n = 1231) followed up in 2016, the prevalence of GDM in 2007 was 362/1231 (29.6%), while the prevalence of pre-pregnancy T2D, was 36/1231 (2.9%). Of the 362 pregnant women with GDM in 2007, 96/362 (26.5%) developed T2D, 142/362 (39.2%) developed impaired fasting glucose, 29/362 (8.0%) developed impaired glucose tolerance, and the remaining 95/362 (26.2%) had normal glycemia in 2016. The prevalence of T2D, based on ADA criteria, stemmed from the prevalence of 36/1231 (2.9%) in 2007 to 141/1231 (11.5%), in 2016. The positive predictive value (PPV) for FBG suggests that, if a woman is positive for GDM in 2007, then the probability of developing T2D in 2016 was about 24%. The opposite is being observed in the predictability of T2D in 2016 using the 2hrBG in diagnosis of GDM in 2007. The PPV value suggests that if a woman was positive for GDM in 2007 then the probability of developing T2D in 2016 was only 3%.

Research conclusions

The results of this study revealed that both raised antenatal FBG and 2hrBG levels could predict postpartum T2D; however, it suggested that each parameter may indicate a distinct T2D pathophysiology. Women with predominant peripheral resistance to the action of insulin, who have raised FBG levels during pregnancy, were at a greater risk of developing T2D, compared with those with raised postprandial 2hrBG levels.

Research perspectives

Our findings suggested that, for women who at a greater risk of developing T2D, postnatal management like frequent follow-ups, lifestyle modifications, and specific treatment protocols, should be applied to slow down the development of T2D and improve the quality of life for them and their newborns.

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ORIGINAL ARTICLE

Retrospective Study

Diabetes patients with comorbidities had unfavorable outcomes following COVID-19: A retrospective study

Shun-Kui Luo, Wei-Hua Hu, Zhan-Jin Lu, Chang Li, Ya-Meng Fan, Qi-Jian Chen, Zai-Shu Chen, Jian-Fang Ye, Shi-Yan Chen, Jun-Lu Tong, Ling-Ling Wang, Jin Mei, Hong-Yun Lu

ORCID number: Shun-Kui Luo 0000-0001-6252-1715; Wei-Hua Hu 0000-0003-4354-3227; Zhan-Jin Lu 0000-0001-9633-0394; Chang Li 0000-0001-7142-8635; Ya-Meng Fan 0000-0002-3166-3144; Qi-Jian Chen 0000-0001-5814-4566; Zai-Shu Chen 0000-0003-0966-8130; Jian-Fang Ye 0000-0003-0515-8627; Shi-Yan Chen 0000-0003-2674-4342; Jun-Lu Tong 0000-0002-9300-3272; Ling-Ling Wang 0000-0001-8066-7638; Jin Mei 0000-0003-4968-0767; Hong-Yun Lu 0000-0001-8794-0887.

Author contributions: Lu HY and Mei J conceptualized the design of the study, had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis; Luo SK and Hu WH drafted the manuscript; Lu ZJ, Luo SK did the analysis, Fan YM reviewed the statistical methods; Li C, Chen QJ, Chen ZS, Fan YM collected the data; Lu ZJ, Ye JF, Chen SY, Wang LL and Tong JL recorded the data.

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Shun-Kui Luo, Zhan-Jin Lu, Jian-Fang Ye, Shi-Yan Chen, Jun-Lu Tong, Department of Endocrinology and Metabolism, the Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, Guangdong Province, China

Wei-Hua Hu, Department of Respiratory Medicine, The First Hospital of Jingzhou, Clinical Medical College, Yangtze University, Jingzhou 434000, Hubei Province, China

Chang Li, Department of Cardiology, Hubei No. 3 People's Hospital of Jianghan University, Wuhan 430033, Hubei Province, China

Ya-Meng Fan, School of Health Sciences, Wuhan University, Wuhan 430071, Hubei Province, China

Qi-Jian Chen, Department of Emergency Medicine, The Fifth Hospital in Wuhan, Wuhan 430050, Hubei Province, China

Zai-Shu Chen, People's Hospital of Jiayu County, Jiayu 437200, Hubei Province, China

Ling-Ling Wang, Department of Gerontology, The Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, Guangdong Province, China

Jin Mei, Anatomy Department, Wenzhou Medical University, Wenzhou 325035, Zhejiang Province, China

Jin Mei, Central Laboratory, Ningbo First Hospital of Zhejiang University, Ningbo 315010, Zhejiang Province, China

Hong-Yun Lu, Department of Endocrinology and Metabolism, Zhuhai Hospital Affiliated with Jinan University, Zhuhai 519000, Guangdong Province, China

Corresponding author: Hong-Yun Lu, MD, PhD, Chief Doctor, Department of Endocrinology and Metabolism, Zhuhai Hospital Affiliated with Jinan University, No. 79 Kangning Road, Zhuhai 519000, Guangdong Province, China. luhongy@mail.sysu.edu.cn

Abstract

BACKGROUND

Previous studies have shown that diabetes mellitus is a common comorbidity of coronavirus disease 2019 (COVID-19), but the effects of diabetes or anti-diabetic

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medication on the mortality of COVID-19 have not been well described.

AIM

To investigate the outcome of different statuses (with or without comorbidity) and anti-diabetic medication use before admission of diabetic after COVID-19.

METHODS

In this multicenter and retrospective study, we enrolled 1422 consecutive hospitalized patients from January 21, 2020, to March 25, 2020, at six hospitals in Hubei Province, China. The primary endpoint was in-hospital mortality. Epidemiological material, demographic information, clinical data, laboratory parameters, radiographic characteristics, treatment and outcome were extracted from electronic medical records using a standardized data collection form. Most of the laboratory data except fasting plasma glucose (FPG) were obtained in first hospitalization, and FPG was collected in the next day morning. Major clinical symptoms, vital signs at admission and comorbidities were collected. The treatment data included not only COVID-19 but also diabetes mellitus. The duration from the onset of symptoms to admission, illness severity, intensive care unit (ICU) admission, and length of hospital stay were also recorded. All data were checked by a team of sophisticated physicians.

RESULTS

Patients with diabetes were 10 years older than non-diabetic patients [(39 - 64) *vs* (56 - 70), $P < 0.001$] and had a higher prevalence of comorbidities such as hypertension (55.5% *vs* 21.4%, $P < 0.001$), coronary heart disease (CHD) (9.9% *vs* 3.5%, $P < 0.001$), cerebrovascular disease (CVD) (3% *vs* 2.2%, $P < 0.001$), and chronic kidney disease (CKD) (4.7% *vs* 1.5%, $P = 0.007$). Mortality (13.6% *vs* 7.2%, $P = 0.003$) was more prevalent among the diabetes group. Further analysis revealed that patients with diabetes who took acarbose had a lower mortality rate (2.2% *vs* 26.1, $P < 0.01$). Multivariable Cox regression showed that male sex [hazard ratio (HR) 2.59 (1.68 - 3.99), $P < 0.001$], hypertension [HR 1.75 (1.18 - 2.60), $P = 0.006$), CKD [HR 4.55 (2.52-8.20), $P < 0.001$], CVD [HR 2.35 (1.27 - 4.33), $P = 0.006$], and age were risk factors for the COVID-19 mortality. Higher HRs were noted in those aged ≥ 65 (HR 11.8 [4.6 - 30.2], $P < 0.001$) *vs* 50-64 years (HR 5.86 [2.27 - 15.12], $P < 0.001$). The survival curve revealed that, compared with the diabetes only group, the mortality was increased in the diabetes with comorbidities group ($P = 0.009$) but was not significantly different from the non-comorbidity group ($P = 0.59$).

CONCLUSION

Patients with diabetes had worse outcomes when suffering from COVID-19; however, the outcome was not associated with diabetes itself but with comorbidities. Furthermore, acarbose could reduce the mortality in diabetic.

Key Words: Diabetes; Coronavirus disease 2019; Mortality; Risk factors; Acarbose

Core Tip: Previous studies have shown that diabetes mellitus is a common comorbidity of coronavirus disease 2019 (COVID-19), but the effects of diabetes or antidiabetic medication on the mortality of COVID-19 have not been well described. This retrospective and multiple-center study investigate the outcome of different statuses (with or without comorbidity) and antidiabetic medication use before admission of diabetic after COVID-19.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) has become an ongoing pandemic and has caused considerable mortality worldwide[1]. Diabetes is a common comorbidity, especially in elderly patients, but the effects of diabetes or anti-diabetic medication on the severity and mortality of COVID-19 have not been well described. As of April 27, 2021, nearly 150 million COVID-19 cases had been confirmed around the world, and more than 3 million patients died of COVID-19 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Well-controlled blood glucose (3.9-10.0 mmol/L) in preexisting diabetes was associated with a significant reduction in the composite adverse outcomes and death of patients with COVID-19[2]. Patients with diabetes often have several comorbidities, and previous research has revealed that hypertension, chronic obstructive pulmonary disease (COPD), chronic kidney disease (CKD), cardiovascular disease and cerebrovascular disease (CVD) are also associated with worse outcomes in patients suffering from COVID-19[3-6]. However, few studies have described the outcome of different comorbidity statuses of patients with diabetes after infection with COVID-19. In addition, few studies have focused on whether anti-diabetic medication would influence the outcome of patients with preexisting diabetes who suffer from COVID-19. Considering this, we performed a multicenter study to investigate the outcome of different statuses (with or without comorbidity) and anti-diabetic medication before admission of patients with diabetes with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.

MATERIALS AND METHODS

Study design and participants

This is a multicenter, observational, retrospective, real-world study that included adult inpatients from six designated tertiary centers (Supplementary Table 1) between January 21 and March 25, 2020. A total of 1422 patients with COVID-19 were screened for this study (Figure 1). All patients were diagnosed with COVID-19 in accordance with WHO interim guidance.

Data collection

Epidemiological material, demographic information, clinical data, laboratory parameters, radiographic characteristics, treatment and outcome were extracted from electronic medical records using a standardized data collection form. Most of the laboratory data except fasting plasma glucose (FPG) were obtained in first hospitalization, and FPG was collected in the next day morning. Major clinical symptoms, vital signs at admission and comorbidities were collected. The treatment data included not only COVID-19 but also diabetes mellitus. The duration from the onset of symptoms to admission, illness severity, intensive care unit (ICU) admission, and length of hospital stay were also recorded. All data were checked by a team of sophisticated physicians.

Diabetes was defined as a history record of diabetes and the use of anti-diabetic medication; otherwise, newly diagnosed diabetes was based on the level of fasting plasma glucose (FPG) (≥ 7.0 mmol/L), random plasma glucose (≥ 11.1 mmol/L), glycosylated hemoglobin (HbA1c) ≥ 6.5% and classic symptoms of hyperglycemia during hospital stay (as the oral glucose tolerance test may lead to hyperglycemia and then to worsening of a COVID-19 patient's illness, it was not used for diagnosis of diabetes in our study[7]). Hypertension was defined by a history of hypertension, the use of anti-hypertensive drugs, or the National Heart Lung and Blood Institute criteria [8]. Coronary heart disease was defined by a history of coronary heart disease. CVD was defined by a history of CVD. ARDS was defined according to the Berlin definition [9]. Acute kidney injury (AKI) was diagnosed according to the KDIGO (Kidney Disease: Improving Global Outcomes) clinical practice guidelines[10]. Acute cardiac injury (ACI) was reported if serum levels of myocardial injury biomarkers were higher than the upper limit of normal[2]. The criteria for classification of COVID-19 severity were according to the Diagnosis and Treatment Protocol for COVID-19 (Trial Version 8)[11]. We divided the patients into two groups: the non-severe group (mild and general types) and the severe group (severe and critical types).

Outcomes

The primary outcome was all-cause mortality after admission. Secondary outcomes were ICU admission and incidence of SARS-CoV-2-related complications, including ARDS, AKI, ACI, secondary infection, shock and hypoproteinemia.

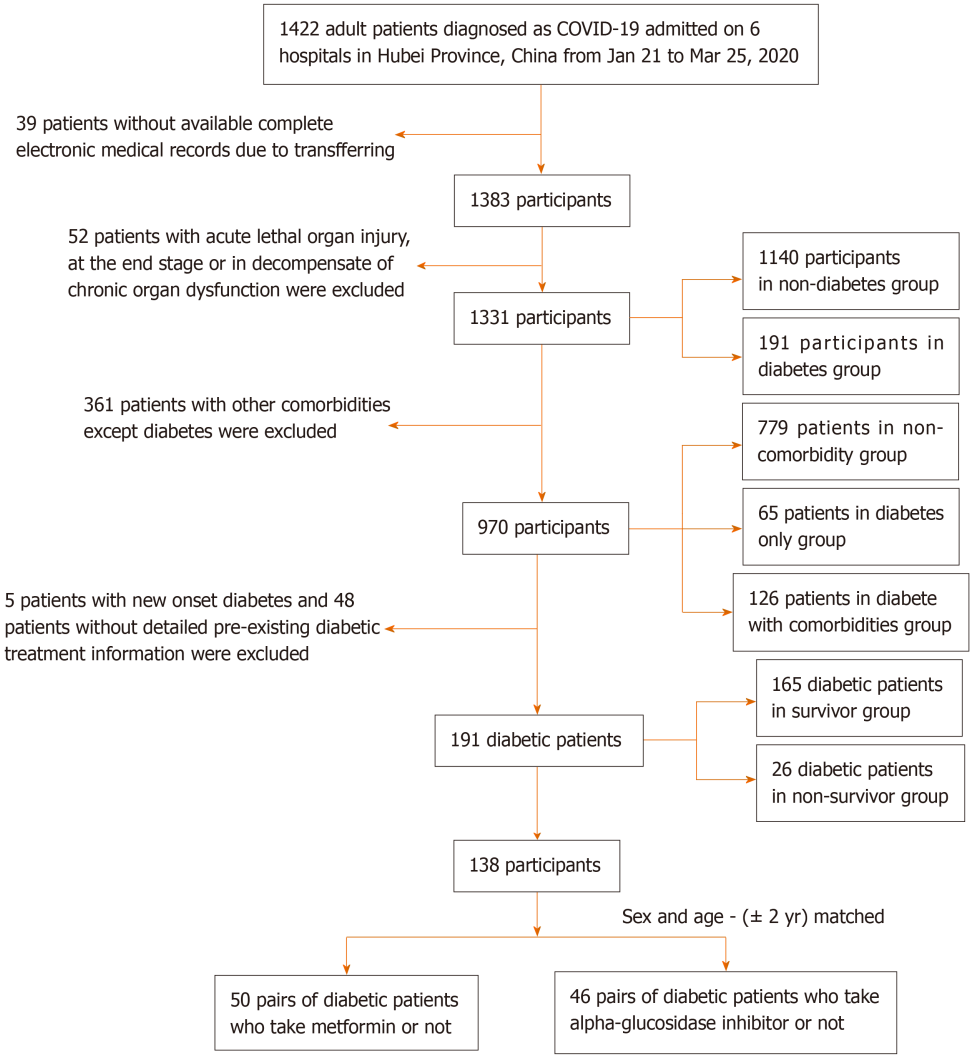

Figure 1 Flow chart of patient recruitment. COVID-19: Coronavirus disease 2019.

Statistical analysis

Continuous variables were described as the mean ± SD or median (IQR). Categorical variables were calculated as frequencies and percentages with available data. The differences in continuous variables among groups were assessed using the independent sample *t*-test or one-way ANOVA for normally distributed continuous variables or the Mann-Whitney *U* test or Kruskal-Wallis *H* test for skewed continuous variables. Pearson's χ^2 test and Fisher's exact test were performed for unordered categorical variables. The Mann-Whitney *U* test or the Kruskal-Wallis *H* test was used for ordered categorical variables. To explore the risk factors associated with mortality, multivariable Cox regression models were performed. The Kaplan-Meier plot was performed to compare the survival probability for the diabetes and non-diabetes groups and among the patients with no comorbidities, only diabetes and diabetes with comorbidities by log-rank test. Additionally, we did not process the missing data. The statistical analyses were conducted with SPSS (version 25.0). A two-sided *P* value less than 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Yameng Fan from Wuhan University.

RESULTS

Clinical characteristics and laboratory results of 1331 patients with COVID-19 divided into different groups

The characteristics of this study population at baseline are given in Table 1. The median age was 54 years old (39-64) and 64 years old (56-70) in the non-diabetes and

Table 1 Baseline characteristics of 1331 coronavirus disease 2019 patients divided into different groups

	Total (*n* = 1331)	Non-diabetes (*n* = 1140)	Diabetes (*n* = 191)	*P*[1] value	Non-comorbidity (*n* = 779)	Diabetes only (*n* = 65)	Diabetes with comorbidities (*n* = 126)	*P*[2] value
Demographic								
Male	673 (50.6)	565 (49.6)	108 (56.5)	0.074	369 (47.4)	40 (61.5)	68 (54.0)	0.046
Age, yr	56.0 (42.0-65.0)	54.0 (39.0-64.0)	64.0 (56.0-70.0)	< 0.001	48.0[c] (36.0-60.0)	57.0 (50.0-64.0)	67.0[c] (59.0-72.0)	< 0.001
18-49	500(37.6)	477 (41.8)	23 (12.0)	<0.001	415 (53.3)[c]	16 (24.6)	7 (5.6)[c]	< 0.001[d]
50-64	458 (34.4)	382 (33.5)	76 (39.8)		253 (32.5)	34 (52.3)	42 (33.3)	
≥ 65	373 (28.0)	281 (24.6)	92 (48.2)		111 (14.2)	15 (23.1)	77 (61.1)	
Wuhan exposure	1190 (89.4)	1008 (88.4)	182 (95.3)	0.004	686 (88.3)	61 (95.3)	120 (95.2)	0.018
Current smoking	107 (8.1)	93 (8.2)	14 (7.4)	0.736	55 (7.2)	3 (4.7)	11 (8.9)	0.149
Onset of symptom, d	8.0 (5.0-14.0)	8.0 (5.0-14.0)	10.0 (6.0-13.0)	0.217	8.0 (4.8-14.0)	10.0 (6.5-16.5)	10.0 (5.8-12.0)	0.109
Symptoms								
Fever	955 (71.8)	823 (72.2)	132 (69.1)	0.381	570 (73.2)	46 (70.8)	86 (68.3)	0.496
Dyspnea	270 (20.3)	227 (19.9)	43 (22.5)	0.408	135 (17.3)	9 (13.8)	34 (27.0)	0.021
Cough	777 (58.4)	660 (57.9)	117 (61.3)	0.383	433 (55.6)	46 (70.8)	71 (56.7)	0.060
Sputum production	138 (10.4)	126 (11.1)	12 (6.3)	0.045	84 (10.8)	4 (6.2)	8 (6.3)	0.175
Hemoptysis	3 (0.2)	3 (0.3)	0 (0.0)	1.000	1 (0.1)	0 (0.0)	0 (0.0)	0.885
Fatigue	362 (27.2)	306 (26.8)	56 (29.3)	0.476	212 (27.2)	16 (24.6)	40 (31.7)	0.489
Headache	47 (3.5)	44 (3.9)	3 (1.6)	0.169	29 (3.7)	3 (4.6)	0 (0.0)	0.010
Nausea or vomiting	44 (3.3)	39 (3.4)	5 (2.6)	0.566	25 (3.2)	2 (3.1)	3 (2.4)	0.939
Diarrhea	112 (8.4)	97 (8.5)	15 (7.9)	0.763	63 (8.1)	9 (13.8)	6 (4.8)	0.091
Temperature, °C	36.8 (36.5-37.5)	36.8 (36.5-37.5)	36.7 (36.4-37.4)	0.018	36.8 (36.5-37.5)	36.8 (36.5-37.6)	36.6 (36.4-37.3)	0.018
≥ 39	30 (2.4)	25 (2.3)	5 (2.7)	0.980	16 (2.2)	2 (3.1)	3 (2.4)	0.889
Pulse ≥ 100 beats per min	244 (18.5)	209 (18.5)	35 (18.3)	0.955	125 (16.1)	12 (18.5)	23 (18.30)	0.761
Blood oxygen saturation < 93%	124 (11.1)	92 (9.6)	32 (19.8)	< 0.001	43 (6.5)	7 (12.5)	25 (23.6)	< 0.001
Respiratory rate > 24 breaths/min	71 (5.4)	56 (5.0)	15 (7.9)	0.105	27 (3.5)	2 (3.1)	13 (10.3)	0.002
Mean systolic blood pressure, mmHg	125 (120-135)	124 (119-135)	128 (120-140)	0.001	121 (118-131)	127 (120-133)	130 (120-140)	< 0.001
Mean diastolic blood pressure, mmHg	80.0 (74.0-85.0)	80.0 (74.0-85.0)	80.0 (74.0-85.0)	0.777	80.0 (73.0-83.0)	80.0 (72.5-85.0)	80.0 (74.0-84.3)	0.550
Radiological findings								
Ground glass opacity	294 (22.1)	265 (23.2)	29 (15.2)	0.013	195 (25.0)	9 (13.8)	20 (15.9)	0.014
Bilateral patchy shadowing	813 (61.1)	687 (60.3)	126 (66.0)	0.134	62 (8.0)	5 (7.7)	4 (3.2)	0.107
Bilateral lesions	962 (82.1)	805 (80.1)	157 (94.0)	< 0.001	524 (76.2)[a]	53 (91.4)	104 (95.4)	< 0.001
Comorbidity								
Hypertension	350 (26.3)	244 (21.4)	106 (55.5)	< 0.001	-	-	-	-
CHD	59 (4.4)	40 (3.5)	19 (9.9)	< 0.001	-	-	-	-
Chronic liver disease	20 (1.5)	18 (1.6)	2 (1.0)	0.812	-	-	-	-

CVD	39 (2.9)	25 (2.2)	14 (7.3)	< 0.001	-	-	-	-
CKD	26 (2.0)	17 (1.5)	9 (4.7)	0.007	-	-	-	-
COPD	10 (0.8)	10 (0.9)	0 (0.0)	0.397	-	-	-	-

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test, One-Way ANOVA or Kruskal-Wallis *H* test as appropriate.
[1]Comparing groups of diabetes and non-diabetes patients.
[2]Comparing groups of non-comorbidity, only diabetes and diabetes with comorbidities.
[d]Mann-Whitney *U* test comparing all subcategories. Compared with diabetes only group.
[a]$P < 0.05$.
[b]$P < 0.05$.
[c]$P < 0.001$.
CHD: Coronary heart disease; CKD: Chronic kidney disease; COPD: Chronic obstructive pulmonary diseas; CVD: Cerebrovascular diseasee.

diabetes groups, respectively. Comorbidities such as hypertension (55.5% *vs* 21.4%), coronary heart disease (9.9% *vs* 3.5%), CVD (7.3% *vs* 2.2%), and CKD (4.7% *vs* 1.5%) were significantly more prevalent in the diabetes group. Mean systolic blood pressure (SBP) was higher in the diabetes group. Moreover, decreased blood oxygen saturation (lower than 93%) occurred more frequently in the diabetes group *vs* the non-diabetes group (19.8% *vs* 19.6%) on admission. Chest CT scan revealed that the incidence of bilateral lesions was higher (94% *vs* 80.1%) in the diabetes group than in the non-diabetes group.

There were numerous differences in laboratory results between the diabetes group and the non-diabetes group with COVID-19 (Table 2). FPG levels were significantly higher in the diabetes group than in the non-diabetes group, as expected, with higher levels of HbA1c. Patients with diabetes had a higher white blood cell count (WBC), neutrophil count (NEU), neutrophil to lymphocyte ratio (NLR), and C-reactive protein (CRP) and a lower lymphocyte count (LY) than the non-diabetic group. These results revealed that diabetes represented more severe inflammation. The percentage of high levels of prothrombin time (PT) and D-dimer among the diabetes group was higher than that among the non-diabetes group. The serum level of albumin (ALB) was lower in the diabetes group than in the non-diabetes group. Meanwhile, urea nitrogen (BUN), a marker of kidney function, was higher in the diabetes group. Non-diabetes participants had significantly lower serum levels of lactate dehydrogenase. Compared with the non-diabetes group, the diabetes group had higher levels of total cholesterol (TCH) and lower high-density lipoprotein cholesterol (HDL-C).

In addition, a between-group comparison with only the diabetes group was performed. The baseline characteristics and radiological findings are also summarized in Table 1. Patients with diabetes with comorbidities were the oldest among the three groups. There was a significant difference in blood oxygen saturation and respiratory rate among the three groups but no significant differences in the comparison of the non-comorbidity group and only diabetes group or the comparison of the diabetes only group and diabetes with comorbidities group. Chest CT scans indicated that the diabetes only group had more incidences of bilateral lesions than the non-comorbidity group.

Although there were numerous differences in laboratory findings among the non-comorbidity group, diabetes only group and diabetes with comorbidities group (Table 2), only ten items had statistical significance between the non-comorbidity group and diabetes only group, including ALB, sodium, BUN, CRP, and HDL-C, as well as FPG and HbA1c, as expected. These results combined with oxygen saturation indicated that there was no difference in cardiac, liver, lung and coagulation function between the groups.

FPG and HbA1c in the diabetes only group and diabetes with comorbidities group were almost at the same level. Compared with the diabetes only group, the diabetes with comorbidities group had a lower LY and a higher NLR and CRP, which represented a more severe inflammatory response.

Treatment and outcome of 1331 patients with COVID-19 divided into different groups

As shown in Table 3, 1223 of the 1331 patients (91.9%) were discharged from the hospital; the rate of mortality of the diabetes group was higher than that of the non-diabetes group (13.6% *vs* 7.2%). Kaplan-Meier survival analysis for all-cause mortality in patients with COVID-19 is shown in Figure 2. The overall survival rate was significantly lower in the diabetes group (log-rank $P < 0.01$, Figure 2A). Compared

Table 2 Laboratory results of 1331 coronavirus disease 2019 patients divided into different groups

	Total (*n* = 1331)	Non-diabetes (*n* = 1140)	Diabetes (*n* = 191)	*P*[1] value	Non-comorbidity (*n* = 779)	Diabetes only (*n* = 65)	Diabetes with comorbidities (*n* = 126)	*P*[2] value
WBC, × 10^9/L	5.42 (4.18-7.10)	5.35 (4.10-6.95)	5.93 (4.49-7.53)	0.003	5.28 (4.00-6.77)	6.11 (4.27-7.68)	5.85 (4.57-7.32)	0.001
NEUT, × 10^9/L	3.58 (2.53-5.12)	3.45 (2.46-5.07)	4.25 (3.13-5.37)	< 0.001	3.29 (2.33-4.64)	4.16 (2.67-5.40)	4.37 (3.20-5.29)	< 0.001
LY, × 10^9/L	1.15 (0.78-1.59)	1.17 (0.80-1.61)	1.04 (0.72-1.43)	0.015	1.25 (0.86-1.65)	1.27 (0.84-1.73)	0.93 (0.68-1.33)[b]	< 0.001
NLR	2.95 (1.97-5.26)	2.79 (1.88-4.93)	3.84 (2.45-6.37)	< 0.001	2.54 (1.79-4.36)	3.15 (2.08-5.06)	4.29 (2.62-7.30)[a]	< 0.001
Hb, g/L	130 (118-140)	130 (118-140)	120 (117-140)	0.195	131 ± 16.3	132 ± 14.4	125 ± 17.6[b]	< 0.001
PLT, × 10^9/L	196 (150-251)	196 (151-251)	196 (147-255)	0.714	196 (152-242)	197 (147-265)	196 (146-255)	0.974
PCT, ng/mL								
< 0.5	981 (94.4)	838 (94.4)	143 (94.7)	0.869	585 (97.3)	53 (100)	90 (91.8)	0.006
≥ 0.5	58 (5.6)	50 (5.6)	8 (5.3)		16 (2.7)	0 (0.0)	8 (8.2)	
CRP	10.9 (1.7-46.7)	9.1 (1.4-39.0)	29.8 (5.3-75.7)	< 0.001	6.11 (1.0-27.7)[a]	13.2 (3.0-61.5)	39.9 (6.6-77.7)[a]	< 0.001
IL-6, pg/mL	2.77 (1.5-14.09)	2.73 (1.5-13.5)	3.09 (1.5-20.4)	0.471	1.80 (1.50-6.27)	2.32 (1.50-5.06)	4.01 (2.72-28.56)	0.008
PT, s	13.0 (11.3-14.9)	12.9 (11.3-14.6)	14.3 (11.9-15.5)	< 0.001	12.80 (11.20-14.30)	13.70 (10.80-15.05)	14.50 (12.35-16.03)	< 0.001
< 16	830 (85.7)	712 (87.1)	118 (78.1)	0.004	493 (87.7)	41 (83.7)	75 (73.5)	0.001
≥ 16	138 (14.3)	105 (12.9)	33 (21.9)		69 (12.3)	8 (16.3)	27 (26.5)	
D-dimer, mg/L	0.49 (0.26-1.14)	0.46 (0.25-1.10)	0.69 (0.35-1.35)	< 0.001	0.38 (0.23-0.80)	0.46 (0.26-0.91)	0.83 (0.46-1.94)[b]	< 0.001
≤ 0.5	555 (52.5)	497 (55.0)	58 (37.9)	< 0.001	386 (63.4)	27 (50.9)	31 (31.0)[b]	< 0.001[3]
> 0.5 to ≤ 1.0	209 (19.8)	163 (18.0)	46 (30.1)		100 (16.4)	18 (34.0)	28 (28.0)	
> 1.0	293 (27.7)	244 (27.0)	49 (32.0)		123 (20.2)	8 (15.1)	41 (41.0)	
ALB, g/L	38.1 ± 5.8	38.5 ± 5.7	35.7 ± 5.5	< 0.001	39.3 ± 5.9[c]	36.5 ± 6.4	35.3 ± 5.0	< 0.001
ALT, U/L	23.1 (14.2-39.0)	23.3 (14.0-40.0)	23.0 (16.0-34.0)	0.844	22.0 (13.8-39.0)	21.0 (16.3-33.5)	24.0 (15.9-34.0)	0.801
AST, U/L	28.8 (22.0-40.4)	28.8 (22.0-40.0)	29.0 (20.0-41.0)	0.583	27.0 (21.0-38.0)	26.0 (18.2-36.5)	31.0 (22.0-43.0)[a]	0.034
ALP, U/L	58.0 (46.0-73.0)	58.0 (46.0-73.0)	55.0 (43.5-74.0)	0.171	58.0 (45.0-72.0)	53.0 (38.0-68.5)	58.0 (45.0-77.0)	0.086
TBIL, mmol/L	10.9 (8.2-14.7)	10.8 (8.2-14.5)	11.4 (8.3-15.7)	0.196	10.8 (8.2-14.7)	11.4 (9.5-14.8)	11.3 (8.0-15.8)	0.429
Potassium, mmol/L	3.90 (3.59-4.20)	3.90 (3.60-4.20)	3.88 (3.52-4.21)	0.325	3.94 ± 0.51	3.94 ± 0.49	3.86 ± 0.62	0.279
Sodium, mmol/L	139 (137-141)	140 (137-141)	138 (136-141)	0.001	140 (138-141)[a]	138 (136-141)	139 (136-142)	0.002
Chlorine ion, mmol/L	104 (102-107)	105 (102-107)	103 (100-106)	0.002	104.2 ± 5.3	103.1 ± 4.5	103.7 ± 5.1	0.218
Calcium, mmol/L	2.11 (2.00-2.21)	2.12 (2.01-2.21)	2.09 (1.95-2.17)	0.005	2.13 ± 0.22	2.11 ± 0.22	2.07 ± 0.18	0.011
Phosphorus, mmol/L	1.03 (0.89-1.19)	1.03 (0.89-1.19)	1.01 (0.73-1.18)	0.359	1.04 (0.90-1.19)	1.03 (0.92-1.17)	1.00 (0.85-1.19)	0.300
BUN, mmol/L	3.96 (3.10-5.25)	3.90 (3.10-5.13)	4.68 (3.60-6.20)	< 0.001	3.70 (2.96-4.66)[a]	4.30 (3.51-5.07)	4.93 (3.60-7.01)	< 0.001

Creatinine, μmol/L	63.6 (53.3-78.0)	63.0 (53.0-77.4)	66.3 (54.0-83.8)	0.088	62.00 (52.70-73.00)	60.00 (52.00-76.60)	67.75 (55.25-90.23)[a]	< 0.001
UA, μmol/L	258 (204-336)	257 (205-336)	258 (193-332)	0.725	253 (203-327)	248 (194-306)	264 (191-352)	0.499
CK, U/L	65.0 (43.0-110)	64.5 (44.0-109)	66.5 (40.3-118)	0.830	62.0 (44.0-98.0)	58.5 (36.8-108)	70.0 (43.8-122)	0.233
LDH, U/L	205 (162-272)	201 (160-261)	229 (180-341)	< 0.001	186 (155-239)	198 (164-282)	251 (195-362)[a]	< 0.001
Hs-cTnI > ULN, pg/mL	130 (22.1)	117 (23.4)	13 (14.90)	0.080	71 (23.5)	3 (12.5)	6 (9.5)	0.027
TG, mmol/L	1.22 (0.92-1.78)	1.20 (0.89-1.77)	1.39 (1.04-1.83)	0.002	1.18 (0.86-1.77)	1.50 (1.05-2.08)	1.36 (1.03-1.79)[a]	0.004
TCH, mmol/L	4.00 (3.40-4.80)	4.01 (3.42-4.80)	4.00 (3.22-4.78)	0.180	4.25 ± 1.09	4.34 ± 1.07	3.88 ± 1.08[a]	0.004
LDL-C, mmol/L	2.50 (3.00-3.12)	2.51 (2.02-3.10)	2.48 (1.87-3.15)	0.368	2.65 ± 0.89	2.76 ± 0.91	2.41 ± 0.87[a]	0.020
HDL-C, mmol/L	1.01 (0.82-1.21)	1.03 (0.84-1.24)	0.91 (0.76-1.08)	< 0.001	1.11 ± 0.42[a]	0.97 ± 0.26	0.92 ± 0.27	< 0.001
FPG, mmol/L	5.80 (5.00-7.46)	5.57 (4.92-6.89)	9.10 (6.50-11.63)	< 0.001	5.37 (4.83-6.50)[c]	9.40 (6.48-11.59)	8.80 (6.50-12.03)	< 0.001
3.9-6.9	693 (69.0)	650 (76.7)	43 (27.6)	< 0.001	475 (80.2)[c]	17 (29.8))	26 (26.3)	< 0.001[3]
7.0-11.1	241 (24.0)	179 (21.1)	62 (39.7)		108 (18.2)	22 (38.6)	40 (40.4)	
≥ 11.1	70 (7.0)	19 (2.2)	51 (32.7)		9 (1.5)	18 (31.6)	33 (33.3)	
HbA1C	6.20 (5.55-7.30)	5.90 (5.40-6.30)	7.87 (6.27-9.03)	< 0.001	5.9 (5.44-6.20)[b]	7.60 (5.64-8.98)	7.89 (6.75-9.21)	< 0.001

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test, One-Way ANOVA or Kruskal-Wallis *H* test as appropriate.

[1]Comparing groups of diabetes and non-diabetes patients.

[2]Comparing groups of non-comorbidity, only diabetes and diabetes with comorbidities.

[3]Mann-Whitney *U* test comparing all subcategories.

Compared with diabetes only group,

[a]$P < 0.05$.

[b]$P < 0.05$.

[c]$P < 0.001$.

ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Urea nitrogen; CK: Creatine kinase; CRP: C reactive protein; FPG: Fasting plasma glucose; Hb: Hemoglobin; HbA1C: Glycosylated hemoglobin; HDL-C: High density lipoprotein cholesterol; Hs-cTnI: Hypersensitive troponin I; LDH: Lactate dehydrogenase; LDL-C: Low density lipoprotein cholesterol; LY: Lymphocyte; NEUT: Neutrophil; NLR: Neutrophil lymphocyte ratio; PCT: Procalcitonin; PLT: Platelet; PT: Prothrombin time; TBIL: Total bilirubin; TCH: Total cholesterol; TG: Triglyceride; UA: Uric acid; WBC: White blood cell.

with non-diabetes patients, more patients with diabetes reported severe cases (34.6% *vs* 21.7%). The diabetes group had a higher rate of ARDS (11% *vs* 5.7%) and hypoproteinemia (15% *vs* 6.5%).

The treatment and primary outcome of the non-comorbidity group and diabetes only group were not different (Table 3), and the results for all-cause mortality were similar in both groups (log-rank $P = 0.59$) (Figure 2B). Regarding the secondary endpoint, there was no difference between the groups except for hypoproteinemia (5.0% *vs* 16.9%). Likewise, there was a similar frequency of COVID-19 pharmacological therapy in the diabetes only patients *vs* diabetes with comorbidities patients; however, the latter was more likely to receive mechanical ventilation (10.8% *vs* 18.3%), had a higher incidence of mortality (4.6% *vs* 18.3%), greater likelihood of shock (0 *vs* 1.6%) and more severe cases (21.5% *vs* 41.3%).

Clinical characteristics and laboratory results of diabetic survivors and non-survivors with COVID-19

Diabetic survivors ($n = 165$) and non-survivors ($n = 26$) shared basic characteristics except for decreased blood oxygen saturation (10.9% *vs* 26.9%) and rapid breathing (18.2% *vs* 26.9%), which were more frequent in non-survivors (Supplementary Table 2), indicating that the latter had severe lung dysfunction. There were numerous

Table 3 Treatments and outcomes of 1331 coronavirus disease 2019 patients divided into different groups

	Tota (*n* = 1331)	Non-diabetes (*n* = 1140)	Diabetes (*n* = 191)	*P*[1] value	Non- comorbidity (*n* = 779)	Diabetes only (*n* = 65)	Diabetes with comorbidities (*n* = 126)	*P*[2] value
Treatments								
Antiviral therapy	1227 (92.2)	1057 (92.7)	170 (89.0)	0.077	725 (93.1)	62 (95.4)	108 (85.7)	**0.010**
Antibiotic therapy	1142 (85.8)	982 (86.1)	160 (83.8)	0.385	665 (85.4)	57 (87.7)	103 (81.7)	0.472
Systemic glucocorticoid	533 (40.0)	458 (40.2)	75 (39.3)	0.813	292 (37.5)	23 (35.4)	52 (41.3)	0.657
Intravenous immunoglobulin	403 (30.3)	342 (30.0)	61 (31.9)	0.590	210 (27.0)	18 (27.7)	43 (34.1)	0.250
Renal replacement therapy	2 (0.2)	1 (0.1)	1 (0.5)	0.267	0 (0.0)	0 (0.0)	1 (0.8)	0.197
Oxygen support								
Oxygenation	786 (59.1)	672 (58.9)	114 (59.7)	0.848	426 (54.7)	38 (58.5)	76 (60.3)	0.446
Mechanical ventilation	154 (11.6)	124 (10.9)	30 (15.7)	0.053	68 (8.7)	7 (10.8)	23 (18.3)	**0.004**
Illness severity								
Severe	313 (23.5)	247 (21.7)	66 (34.6)	**< 0.001**	123 (15.8)	14 (21.5)	52 (41.3)[a]	**< 0.001**
Complications								
ARDS	86 (6.5)	65 (5.7)	21 (11.0)	**0.006**	26 (3.3)	2 (3.1)	19 (15.1)	**< 0.001**
ACI	148 (11.1)	132 (11.6)	16 (8.4)	0.193	77 (9.9)	3 (4.6)	12 (9.5)	0.379
AKI	18 (1.4)	14 (1.2)	4 (2.1)	0.535	6 (0.8)	1 (1.5)	3 (2.4)	0.122
Secondary infection	161 (12.1)	139 (12.2)	22 (11.5)	0.791	76 (9.8)	4 (6.2)	18 (14.3)	0.162
Shock	25 (1.9)	23 (2.0)	2 (1.0)	0.531	9 (1.2)	0 (0.0)	2 (1.6)[a]	0.706
Hypoproteinemia < 30g/l	99 (7.7)	71 (6.5)	28 (15.0)	**< 0.001**	38 (5.0)[c]	11 (16.9)	17 (13.9)	**< 0.001**
Length of hospital stay, d	17.0 (10.0-24.0)	17.0 (10.0-24.0)	16.0 (10.0-25.0)	0.655	17.0 (11.0-24.0)	19.0 (11.5-27.0)	16.0 (8.0-22.5)	0.109
ICU admission	125 (9.4)	103 (9.0)	22 (11.5)	0.276	57 (7.3)	5 (7.7)	17 (13.5)	0.062
Duration from admission to ICU, d	4.00 (1.00-7.50)	5.00 (1.00-8.00)	3.50 (1.75-5.25)	0.383	4.50 (1.00-8.00)	5.00 (1.50-6.00)	3 (1.50-4.50)	0.733
Prognosis								
Death, No	108 (8.1)	82 (7.2)	26 (13.6)	**0.003**	26 (3.3)	3 (4.6)	23 (18.3)	**< 0.001**

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test, One-Way ANOVA or Kruskal-Wallis *H* test as appropriate.

[1]Comparing groups of diabetic and non-diabetic patients.

[2]Comparing groups of non-comorbidity, only diabetes and diabetes with comorbidities.

Compared with diabetes only group:

[a]$P < 0.05$.

[b]$P < 0.05$.

[c]$P < 0.001$.

ACI: Acute cardiac injury; AKI: Acute kidney injury; ARDS: Acute respiratory distress syndrome.

differences in laboratory results between diabetic survivors and non-survivors with COVID-19 that reflected the functions of different organs and systems (Supplementary Table 2). Diabetic non-survivors had higher WBC, NEU, NLR, CRP, and IL-6 and lower LY, reflecting that mortality patients had severe inflammatory responses. Serum levels of PT, D-dimer, ALT, AST, BUN, creatinine, CK, and LDH were all significantly higher in non-survivors (Table 4), which reflected more severe coagulation, liver, kidney, and cardiac dysfunction. Diabetic non-survivors reported higher average FPG compared with survivors.

Table 4 Laboratory results of diabetic survivors and non-survivors with coronavirus disease 2019

	Total (*n* = 191)	Survivors (*n* = 165)	Non-survivors (*n* = 26)	*P* value
WBC, × 10^9per L	5.94 (4.49-7.53)	5.91 (4.42-7.29)	7.26(5.19-13.07)	0.016
NEUT, × 10^9per L	4.25 (3.13-5.37)	4.09 (3.01-5.13)	6.22 (3.69-11.33)	< 0.001
LY, × 10^9per L	1.04 (0.72-1.43)	1.08 (0.78-1.48)	0.65 (0.56-1.07)	< 0.001
NLR	3.85 (2.45-6.37)	3.50 (2.33-5.53)	10.43 (5.78-16.84)	< 0.001
Hb, g/L	127.3 ± 16.9	126.8 ± 16.7	131.6 ± 18.3	0.314
PLT, × 10^9per L	196 (147-255)	201 (152-201)	155 (110-230)	0.033
PCT, ng/mL				
< 0.5	143 (94.7)	132 (98.5)	11 (64.7)	< 0.001
≥ 0.5	8 (5.3)	2 (1.5)	6 (35.3)	
CRP	29.8 (5.5-75.9)	25.4 (4.4-63.0)	115.3 (66.1-170.6)	< 0.001
IL-6, pg/mL	3.31 (1.64-17.49)	3.09 (1.50-5.25)	83.47 (35.75-243.60)	< 0.001
PT, s	14.30 (11.90-15.50)	14.00 (11.60-15.40)	16.20 (13.52-18.92)	0.002
< 16	116 (76.8)	110 (81.5)	6 (37.5)	< 0.001
≥ 16	35 (23.2)	25 (18.5)	10 (62.5)	
D-dimer, mg/L	0.69 (0.35-1.35)	0.62 (0.62-1.09)	5.40 (1.50-21.00)	< 0.001
≤ 0.5	58 (37.9)	57 (41.9)	1 (5.9)	< 0.001
> 0.5 to ≤ 1.0	46 (30.1)	43 (31.6)	3 (17.6)	
> 1.0	49 (32.0)	36 (26.5)	13 (76.5)	
ALB, g/L	35.7 ± 5.5	36.0 ± 30.5	33.5 ± 23.4	0.031
ALT, U/L	23.0 (16.0-34.0)	21.3 (15.3-32.3)	31.0 (20.9-46.6)	0.008
AST, U/L	29.0 (20.0-41.0)	27.0 (19.0-38.7)	43.0 (31.0-60.5)	< 0.001
ALP, U/L	55.0 (43.5-74.0)	55.0 (41.5-73.0)	57.0 (49.5-89.5)	0.241
TBIL, mmol/L	11.3 (8.3-15.7)	11.4 (9.0-15.1)	11.2 (7.6-28.0)	0.642
Potassium, mmol/L	3.88 (3.52-4.21)	3.90 (3.54-4.21)	3.65 (3.37-4.30)	0.381
Sodium, mmol/L	138.4 ± 4.3	138.2 ± 3.9	139.3 ± 6.4	0.418
Chlorine ion, mmol/L	103.5 ± 4.9	103.2 ± 4.7	105.3 ± 6.0	0.052
Calcium, mmol/L	2.09 (1.95-2.17)	2.10 (1.95-2.20)	2.00 (1.89-2.11)	0.042
Phosphorus, mmol/L	1.01 (0.86-1.18)	1.02 (0.87-1.19)	0.93 (0.76-1.18)	0.268
BUN, mmol/L	4.70 (3.60-6.22)	4.5 (3.59-5.82)	6.51 (4.92-17.45)	< 0.001
Creatinine, μmol/L	66.3 (54.0-83.8)	64.0 (44.6-81.0)	73.0 (64.0-129.6)	0.006
UA, μmol/L	258 (193-332)	258 (147-321)	293 (179-428)	0.286
CK, U/L	66.5 (40.3-117.8)	61.0 (36.5-111.0)	85.0 (71.0-364.0)	0.002
LDH, U/L	229 (180-341)	216 (172-219)	522 (420-611)	< 0.001
Hs-cTnI > ULN, pg/mL	10/88 (11.4)	8/72 (11.1)	2/16 (12.5)	1.000
TG, mmol/L	1.39 (1.04-1.83)	1.41 (1.05-1.98)	1.31 (0.99-1.57)	0.398
TCH, mmol/L	4.04 ± 1.10	4.12 ± 1.06	3.39 ± 1.16	0.009
LDL-C, mmol/L	2.54 ± 0.90	2.59 ± 0.88	2.10 ± 0.93	0.036
HDL-C, mmol/L	0.94 ± 0.26	0.94 ± 0.26	0.89 ± 0.33	0.450
FPG, mmol/L	9.10 (6.50-11.72)	8.70 (6.50-11.36)	12.00 (9.40-16.81)	0.011
3.9-6.9	43 (27.7)	40 (28.6)	3 (20.0)	0.069[1]
7.0-11.1	61 (39.4)	58 (41.4)	3 (20.0)	

≥ 11.1	51 (32.9)	42 (30.0)	9 (60.0)	
HbA1C	7.77 ± 1.97	7.61 ± 1.90	9.53 ± 2.02	0.021

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test as appropriate. [1]Mann-Whitney *U* test comparing all subcategories.

P: Comparing groups of diabetic survivors and non-survivors; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Urea nitrogen; CK: Creatine kinase; CRP: C reactive protein; FPG: Fasting plasma glucose; Hb: Hemoglobin; HbA1C: Glycosylated hemoglobin; HDL-C: High density lipoprotein cholesterol; Hs-cTnI: Hypersensitive troponin I; LDH: Lactate dehydrogenase; LDL-C: Low density lipoprotein cholesterol; LY: Lymphocyte; NEUT: Neutrophil; NLR: Neutrophil lymphocyte ratio; PCT: Procalcitonin; PLT: Platelet; PT: Prothrombin time; TBIL: Total bilirubin; TCH: Total cholesterol; TG: Triglyceride; UA: Uric acid; WBC: White blood cell.

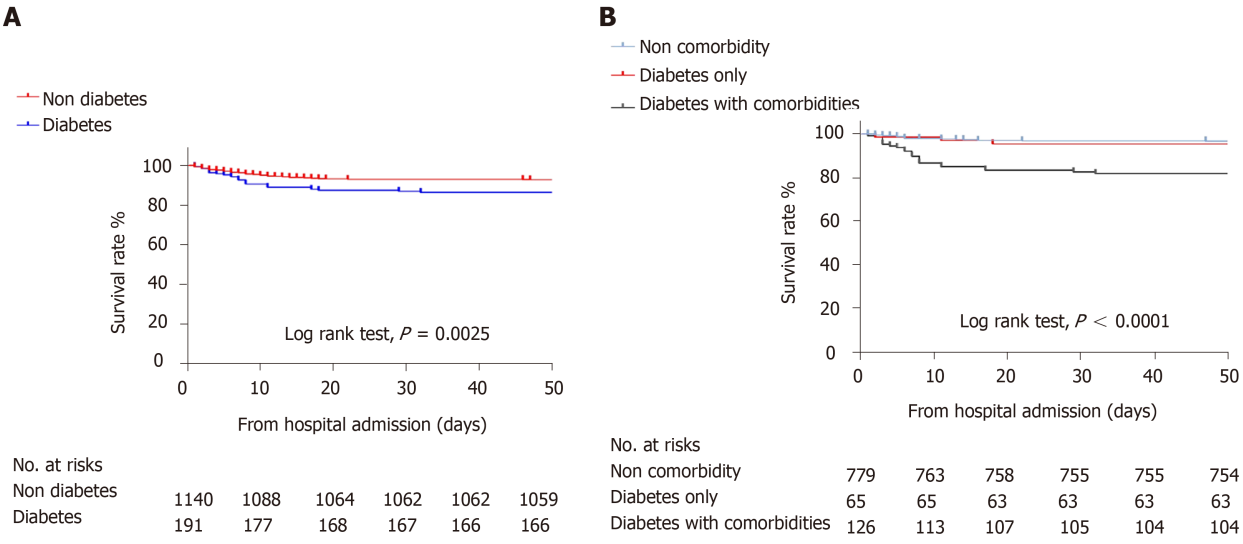

Figure 2 Kaplan-Meier survival curves of in-hospital mortality among patients with coronavirus disease 2019. A: Kaplan-Meier survival curves for in-hospital mortality between diabetes and non-diabetes patients from hospital admission. B: Kaplan-Meier survival curves for in-hospital mortality comparison of patients without comorbidities, diabetes only and diabetes with comorbidities from hospital admission. Patients without comorbidities and diabetes were compared only from hospital admission (log rank test, *P* = 0.590). Patients with only diabetes and diabetes with comorbidities from hospital admission were compared (log rank test, *P* = 0.009).

Treatment and outcome of diabetic survivors and non-survivors with COVID-19

Undoubtedly, higher proportions of complications, including ARDS (3.0 *vs* 61.5%), ACI (5.5% *vs* 26.9%), shock (0 *vs* 11.5%), secondary infection (6.1% *vs* 46.2%), AKI (0.6% *vs* 7.7%) and coagulopathy (15.8% *vs* 38.5%), were found in non-survivors (Table 5). Likewise, the non-survivor group had a greater incidence of severe cases (33.7% *vs* 100%) and ICU admission (6.7% *vs* 42.3%) and was more likely to receive corticosteroids (33.3% *vs* 73.1%). There was a significantly lower frequency of hypoglycemic medication in diabetic non-survivors *vs* diabetic survivors, including metformin (30.9% *vs* 11.5%), sulfonylurea (21.8% *vs* 3.8%) and acarbose (45.5% *vs* 7.7%), which might be related to controlled blood glucose.

Clinical characteristics, laboratory results, treatment and outcome of patients with diabetes with COVID-19 using metformin and matched non-metformin users

Of 191 patients with diabetes with COVID-19, 54 cases were using metformin, and after sex and age matching, there were 50 patients using metformin and 50 sex- and age-matched non-metformin users. The frequency of fever (54% *vs* 78%) and fatigue (38% *vs* 18%) showed significant differences in clinical characteristics between patients with diabetes with COVID-19 using metformin and matched non-metformin users (Supplementary Table 3). Laboratory findings (Table 6) revealed that metformin users had lower levels of LDH and FPG; however, the distribution of glucose was similar. The results that referred to liver, kidney, cardiac, coagulation and inflammatory response function were not statistically significant. The primary outcome and secondary outcome of patients who used metformin were comparable to matched non-metformin users (Table 7). The former group showed a higher need for antivirals (98% *vs* 84%) and antibiotics (90% *vs* 74%). Insulin (52.0% *vs* 20%), sulfonylurea (36.0% *vs*

Table 5 Treatments and outcomes of diabetic survivors and non-survivors with coronavirus disease 2019

	Total (*n* = 191)	Survivors (*n* = 165)	Non-survivors (*n* = 26)	*P* value
Treatments				
Antiviral therapy	170 (89.0)	149 (90.3)	21 (80.8)	0.268
Antibiotic therapy	160 (83.8)	139 (84.2)	21 (80.8)	0.873
Systemic glucocorticoids	74 (38.7)	55 (33.3)	19 (73.1)	**< 0.001**
Intravenous immunoglobulin	60 (31.4)	50 (30.3)	10 (38.5)	0.405
Renal replacement therapy	1 (0.5)	0 (0.0)	1 (3.8)	0.136
Insulin	88 (46.1)	75 (45.5)	13 (50.0)	0.666
Metformin	54 (28.3)	51 (30.9)	3 (11.5)	**0.041**
Sulfonylurea	37 (19.4)	36 (21.8)	1 (3.8)	**0.031**
DPP-4 inhibitor	11 (5.8)	10 (6.1)	1 (3.8)	1.000
Acarbose	77 (40.3)	75 (45.5)	2 (7.7)	**< 0.001**
Thiazolidinedione	7 (3.7)	7 (4.2)	0 (0.0)	0.596
Oxygen support				
Oxygenation	115 (60.2)	95 (57.6)	20 (76.9)	0.061
Mechanical ventilation	35 (18.3)	15 (9.1)	20 (76.9)	**< 0.001**
Illness severity				
Severe	63 (33.0)	37 (33.7)	26 (100)	**< 0.001**
Complications				
ARDS	21 (11.0)	5 (3.0)	16 (61.5)	**< 0.001**
ACI	16 (8.4)	9 (5.5)	7 (26.9)	**0.001**
AKI	3 (1.6)	1 (0.6)	2 (7.7)	**0.049**
Secondary infection	22 (11.5)	10 (6.1)	12 (46.2)	**< 0.001**
Shock	3 (1.6)	0 (0.0)	3 (11.5)	**0.002**
Hypoproteinemia < 30 g/L	28 (14.7)	22 (13.3)	6 (23.1)	0.314
Coagulopathy	36 (18.8)	26 (15.8)	10 (38.5)	**0.013**
Length of hospital stay, d	16.0 (10.0-25.0)	18.0 (11.5-26.0)	7.0 (3.0-11.0)	**< 0.001**
ICU admission	22 (11.5)	11 (6.7)	11 (42.3)	**< 0.001**
Duration from admission to ICU, d	4.00 ± 3.51	3.91 ± 3.11	4.09 ± 4.01	0.907

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test as appropriate. Comparing groups of diabetic survivors and non-survivors. ACI: Acute cardiac injury; AKI: Acute kidney injury; ARDS: Acute respiratory distress syndrome.

2%), acarbose (56.0% *vs* 6%), and thiazolidinedione (12% *vs* 0) were also applied significantly more frequently to the individuals using metformin.

Clinical characteristics, laboratory results, treatment and outcome of patients with diabetes with COVID-19 using acarbose and matched non-acarbose users

Of 191 patients with diabetes with COVID-19, 77 cases were treated with acarbose, and after sex and age matching, there were 46 patients treated with acarbose and 46 sex- and age-matched non-acarbose users. Supplementary Table 3 shows that the length of symptom onset to hospital admission was longer in the acarbose group than in the matched non-acarbose group, which indicated that the symptoms in the former patients might be relatively mild. Notably, some inflammatory response-related laboratory results, such as WBC, NLR, and CRP, were significantly lower in the acarbose group (Table 6). Furthermore, these differences were not related to glucose control, as the serum level of glucose in both groups was comparable.

Table 6 Laboratory results of diabetic coronavirus disease 2019 patients using metformin or acarbose and matched non-metformin or non-acarbose inhibitor user

	Metformin (*n* = 50)	Matched non-Metformin (*n* = 50)	Acarbose (*n* = 46)	Matched non-acarbose (*n* = 46)
WBC, × 10^9/L	6.33 ± 2.25	6.27 ± 2.62	4.83 (4.04-6.68)	5.91 (4.42-9.35)[c]
NEUT, × 10^9/L	4.20 (3.02-5.18)	4.17 (3.21-5.87)	3.50 (2.48-4.74)	4.60 (3.14-8.13)
LY, × 10^9/L	1.20 (0.69-1.74)	1.14 (0.82-1.50)	1.19 ± 0.55	1.04 ± 0.53
NLR	3.69 (2.11-6.05)	3.74 (2.47-5.55)	3.25 (2.05-4.41)	4.88 (2.50-12.32)[d]
Hb, g/L	126.0 ± 15.7	126.5 ± 14.9	126.0 ± 16.9	129.3 ± 17.3
PLT, × 10^9/L	229.5 ± 93.5	208.5 ± 103.8	233.0 ± 93.2	214.2 ± 99.7
PCT, ng/mL				
< 0.5	43 (100.0)	34 (91.9)	37 (100.0)	34 (87.2)
≥ 0.5	0 (0.0)	3 (8.1)	0 (0.0)	5 (12.8)
CRP	50.7 (5.0-78.0)	46.5 (6.3-106.8)	26.2 (3.7-52.2)	63.8 (10.8-83.4)[c]
IL-6, pg/mL	2.07 (1.50-4.90)	3.20 (1.68-67.28)	2.58 (1.50-5.06)	19.88 (1.95-67.28)
PT, s	13.5 ± 2.7	14.2 ± 2.2	14.1 ± 2.6	14.2 ± 3.2
< 16	37 (86.0)	31 (79.5)	30 (78.9)	27 (73.0)
≥ 16	6 (14.0)	8 (20.5)	8 (21.1)	10 (27.0)
D-dimer, mg/L	0.45 (0.26-1.19)	0.83 (0.33-1.60)	0.59 (0.33-0.98)	0.96 (0.39-5.40)
≤ 0.5	22 (52.4)	13 (33.3)	18 (42.9)	12 (33.3)
> 0.5 to ≤ 1.0	6 (14.3)	11 (28.2)	15 (35.7)	7 (19.4)
> 1.0	14 (33.3)	15 (38.5)	9 (21.4)	17 (47.2)
ALB, g/L	35.7 ± 5.9	35.8 ± 5.5	35.7 ± 6.5	35.1 ± 4.8
ALT, U/L	20.0 (13.5-27.5)	22.0 (17.0-36.0)	20.0 (14.0-31.0)	23.00 (14.00-33.25)
AST, U/L	25.5 (18.5-33.7)	29.0 (20.0-42.0)	23.0 (17.5-36.4)	31.0 (21.5-39.6)
ALP, U/L	51.0 (37.0-71.0)	54.0 (44.0-68.0)	59.5 24.6	61.5 25.5
TBIL, mmol/L	12.2 ± 5.4	13.4 ± 16.2	11.5 ± 4.8	13.5 ± 5.4
Potassium, mmol/L	3.81 ± 0.46	3.77 ± 0.54	3.90 ± 0.50	3.82 ± 0.63
Sodium, mmol/L	137.9 ± 3.9	138.2 ± 4.1	138. 5 ± 3.8	138.1 ± 4.7
Chlorine ion, mmol/L	103.3 ± 4.5	103.4 ± 5.0	103.2 ± 4.6	103.7 ± 5.3
Calcium, mmol/L	2.14 ± 0.22	2.05 ± 0.18[a]	2.12 ± 0.22	2.08 ± 0.19
Phosphorus, mmol/L	1.02 (0.83-1.21)	0.99 (0.87-1.17)	1.07(0.88-1.21)	1.00 (0.77-1.21)
BUN□mmol/L	4.40 (3.67-4.84)	5.20 (3.50-5.75)	4.16 (3.60-5.18)	5.04 (3.80-6.64)
Creatinine, μmol/L	54.0 (49.0-73.7)	60.0 (52.5-90.3)	59.5 (48.8-74.5)	68.0 (55.0-89.3)
UA, μmol/L	266.5 ± 96.6	260.9 ± 98.7	229 (168-263)	258 (179-324)
CK, U/L	64.0 (49.0-84. 0)	82.0 (39.0-135.3)	53.5 (35.5-73.8)	71.0 (40.0-114.0)
LDH, U/L	237 ± 115	304 ± 162[a]	229 (185-263)	267 (181-446)
Hs-cTnI > ULN , pg/mL	0/24 (0.0)	3/15 (20.0)	1/21 (4.8)	2/26 (7.7)
TG, mmol/L	1.55 (1.15-1.82)	1.32 (1.08-3.40)	1.36 (1.05-1.83)	1.15 (0.94-1.61)
TCH, mmol/L	3.91 ± 0.87	3.80 ± 0.92	4.40 ± 1.14	4.04 ± 0.96
LDL-C, mmol/L	2.40 ± 0.73	2.43 ± 0.78	2.84 ± 0.87	2.57 ± 0.83
HDL-C, mmol/L	0.93 ± 0.22	0.88 ± 0.28	0.98 ± 0.27	0.93 ± 0.23
FPG, mmol/L	10.57 ± 4.92	8.32 ± 2.47[b]	9.92 ± 4.90	10.00 ± 4.26

3.9-6.9	11 (44.0)	13 (38.2)	12 (30.8)	8 (22.2)
7.0-11.1	13 (52.0)	21 (61.8)	13 (33.3)	16 (44.4)
≥ 11.1	1 (4.0)	0 (0.0)	14 (35.9)	12 (33.3)
HbA1C	7.96 ± 1.85	6.71 ± 1.94	7.85 ± 1.78	8.25 ± 2.04

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test as appropriate.
Comparison of metformin users and non-users:
[a]$P < 0.05$.
[b]$P < 0.01$.
Comparison of acarbose users and non-users:
[c]$P < 0.05$.
[d]$P < 0.01$.
ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Urea nitrogen; CK: Creatine kinase; CRP: C reactive protein; FPG: Fasting plasma glucose; Hb: Hemoglobin; HbA1C: Glycosylated hemoglobin; HDL-C: High density lipoprotein cholesterol; Hs-cTnI: Hypersensitive troponin I; LDH: Lactate dehydrogenase; LDL-C: Low density lipoprotein cholesterol; LY: Lymphocyte; NEUT: Neutrophil; NLR: Neutrophil lymphocyte ratio; PCT: Procalcitonin; PLT: Platelet; PT: Prothrombin time; TBIL: Total bilirubin; TCH: Total cholesterol; TG: Triglyceride; UA: Uric acid; WBC: White blood cell.

The mortality rate (2.2% *vs* 26.1%) was lower in the acarbose group (Table 7), as were the rates of ARDS (2.2% *vs* 17.4%) and shock (2.2% *vs* 21.7%). At the same time, patients who were treated with acarbose indicated a lower need for treatment with corticosteroids (26.1% *vs* 47.8%), immunoglobin (23.9% *vs* 47.8%), mechanical ventilation (6.5% *vs* 21.7%), and insulin (50.0% *vs* 84.8%).

Independent risk factors for mortality of patients with COVID-19

Among the 1131 included patients, multivariable Cox regression (Table 8) showed that male [hazard ratio (HR) 2.59, 95%CI 1.63-3.99], hypertension (HR 1.75, 95%CI 1.18-2.6), CKD (HR 4.55, 95%CI 2.52-8.20), and CVD (HR 2.35, 95%CI 1.27-4.33) were risk factors for COVID-19 mortality. Age was also a risk factor for COVID-19 mortality. However, diabetes alone was not an independent risk factor for mortality in patients with COVID-19.

DISCUSSION

A number of studies have demonstrated that patients with diabetes have a higher risk of mortality from COVID, as well as a greater risk of developing more severe cases[4,7,12,13]. Guo *et al*[13] reported that diabetes was a risk factor for the progression and prognosis of COVID-19. However, Shi *et al*[14] pointed out that diabetes was not independently associated with COVID mortality, while commonalities, such as hypertension and cardiovascular disease, played more important roles in contributing to the in-hospital death of patients with COVID-19, which was relatively limited in size. In this study, which had relatively rich clinical data, we found that diabetes alone was not an independent risk factor for in-hospital mortality from COVID-19, but comorbidities such as hypertension and CKD were risk factors; this result was consistent with a previous study[14]. Partially consistent with previous studies, our study found that compared with non-diabetic patients, patients with diabetes with COVID-19 were older, had worse outcomes, including a higher rate of mortality, severe cases and ARDS, and presented severe inflammatory response, lung and coagulation dysfunction[7,13,15]. In this study, up to 88% of diabetic patients were greater than or equal to 50 years of age, more over, older age was an independent risk factor of mortality in COVID-19, which was consistent with previous studies[3,14]. Additionally, patients with diabetes had increased levels of urea nitrogen and decreased levels of albumin. These abnormalities indicated that COVID-19 may be associated with progressive organ injury in patients with diabetes. Preexisting hypertension, CHD, CVD, and CKD had higher frequencies in the diabetic group. Recent studies reported that patients with cardiovascular hypertension, CKD, and CVD were more likely to develop severe cases[4,6,16], so we compared patients with diabetes and COVID-19 without comorbidity and patients with COVID-19 without any comorbidity to identify whether diabetes without comorbidity was a risk factor for COVID-19. In our study, there was no difference in the outcome between the non-comorbidity group and the diabetes only group. Shi *et al*[16] reported that even though

Table 7 Treatments and outcomes of diabetic coronavirus disease 2019 patients using metformin and matched non-metformin, acarbose and matched non-acarbose

	Metformin (*n* = 50)	Matched non-Metformin (*n* = 50)	Acarbose (*n* = 46)	Matched non-acarbose (*n* = 46)
Treatments				
Antiviral therapy	49 (98.0)	42 (84.0)[a]	41 (89.1)	43 (93.5)
Antibiotic therapy	45 (90.0)	37 (74.0)[a]	40 (87.0)	40 (87.0)
systemic glucocorticoids	17 (34.0)	16 (32.0)	12 (26.1)	22 (47.8)[e]
Intravenous immunoglobulin	15 (30.0)	11 (22.0)	11 (23.9)	22 (47.8)[e]
Renal replacement therapy	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)
Insulin	26 (52.0)	10 (20.0)[b]	23 (50.0)	39 (84.8)[g]
Metformin	50	0	21 (45.7)	15 (32.6)
Sulfonylurea	18 (36.0)	1 (2.0)[c]	17 (37.0)	8 (17.4)[e]
DPP-4 inhibitor	3 (6.0)	0 (0.0)	3 (6.5)	3 (6.5)
Acarbose	28 (56.0)	3 (6.0)[c]	46 (100.0)	0 (0.0)
thiazolidinedione	6 (12.0)	0 (0.0)[a]	4 (8.7)	0 (0.0)
Oxygen support				
Oxygenation	32 (64.0)	21 (42.0)	30 (65.2)	30 (65.2)
Mechanical ventilation	6 (12.0)	11 (22.0)	3 (6.5)	10 (21.7)[e]
Illness severity				
Severe	14 (28.0)	21 (42.0)	12 (26.1)	18 (39.1)
Complications				
ARDS	4 (8.0)	8 (16.0)	1 (2.2)	8 (17.4)[e]
ACI	1 (2.0)	4 (8.0)	2 (4.3)	6 (13.0)
AKI	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.3)
Secondary infection	8 (16.0)	5 (10.0)	0 (0.0)	2 (4.3)
Shock	1 (2.0)	0 (0.0)	1 (2.2)	10 (21.7)[e]
Hypoproteinemia < 30 g/L	9 (18.0)	5 (10.0)	10 (21.7)	6 (13.0)
Coagulopathy	6 (12.0)	9 (18.0)	8 (17.4)	10 (21.7)
Length of hospital stay, d	17.60 ± 8.74	16.80 ± 10.51	18.37 ± 8.15	16.52 ± 9.96
ICU admission	6 (12.0)	6 (12.0)	3 (6.5)	8 (17.4)
Duration from admission to ICU, d	3.83 ± 2.04	2.83 ± 2.14	6.00 (3.50-6.00)	2.50 (2.00-5.00)
Prognosis				
Discharged	47 (94)	41 (82)	45 (97.8)	34 (73.9)[f]
Death	3 (6.0)	9 (18)	1 (2.2)	12 (26.1)

Data are expressed as *n* (%), mean ± SD or median (IQR). *P* values were calculated by *t* Test, Mann-Whitney *U* test, χ^2 test, Fisher's exact test as appropriate.
Comparison of metformin users and non-users:
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
Comparison of acarbose users and non-users:
[e]$P < 0.05$.
[f]$P < 0.01$.
[g]$P < 0.001$.
ACI: Acute cardiac injury; AKI: Acute kidney injury; ARDS: Acute respiratory distress syndrome.

patients with COVID-19 with diabetes had worse outcomes, it was not independently

Table 8 Multivariate COX regression analysis on the risk factors associated with mortality of 1331 coronavirus disease 2019 patients

Factor	Hazard ratio	*P* value
Sex (male)	2.59 (1.68-3.99)	< 0.001
Age, yr		
18-49	1 (ref)	
50-64	5.86 (2.27-15.12)	< 0.001
≥ 65	11.8 (4.6- 30.2)	< 0.001
Hypertension	1.75 (1.18-2.60)	0.006
CKD	4.55 (2.52-8.20)	< 0.001
CVD	2.35 (1.27-4.33)	0.006
Diabetes	0.98 (0.62-1.54)	0.918

CKD: Chronic kidney disease; CVD: Cerebrovascular disease.

associated with in-hospital death, which was consistent with our results. In addition, most laboratory results were comparable between the non-comorbidity group and the diabetes only group, except for CRP, albumin, sodium, urea nitrogen, HDL-C and, of course, blood glucose. CRP is an inflammatory biomarker that is related to glucose homoeostasis, obesity and atherosclerosis[17] and was independently related to insulin sensitivity[18]. In addition, insulin resistance was a main characteristic of type 2 diabetes; since CRP was related to the chronic inflammatory situation, and the levels of WBC, NEU, and LY, which reflected the acute infection with the disease pathogen, were not statistically significant, we inferred that diabetes itself did not increase the degree of inflammation after SARS-CoV-2 infection.

Patients with diabetes with comorbidities were more seriously ill when compared with the diabetes only group and non-comorbidity group. The mortality was higher in the diabetes with comorbidities group, but the difference between both diabetes groups had no relation to FPG because the median FPG in both diabetes groups was comparable. Patients with diabetes with comorbidity were 10 years older than patients who had no comorbidity except diabetes; furthermore, age ≥ 65 years was associated with a greater risk of death[4]. As described above, patients with hypertension and CVD were more likely to develop severe cases[4]. Furthermore, our analysis indicated that age, hypertension, CKD, and CVD were risk factors for COVID-19 mortality. Since the diabetes with comorbidities group had a higher prevalence of hypertension, CKD and CVD, there was no doubt that patients with diabetes with comorbidities had worse outcomes.

Comparing to the survivor of diabetic patients with COVID-19, the diabetic patients who died of COVID-19 had more severe inflammatory response, progressive organ injury, and also, undoubtedly, higher proportions of complications and severe cases. Randomised Evaluation of COVID-19 Therapy (RECOVERY) Collaborative Group[19] and WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group[20] reported that systemic glucocorticoids was conducive to the reduction in mortality of COVID-19 severe cases. As the percentage of severe case in non-survivor group were 100%, while that rate in survivor group was just 33%, there was no doubt that non-survivor group had higher rate of using systemic glucocorticoids. Therefore, in such cases, higher rate of systemic glucocorticoids treatment in non-survivor group did not indicated higher rate of mortality in systemic glucocorticoids treatment.

One unanticipated result was that acarbose, not metformin, could improve prognosis through a decrease in the degree of inflammation, which was independent of the blood glucose level. In addition, acarbose accounted for 97% of the glycosidase inhibitors used. Feng *et al*[21] reported that acarbose could effectively block the metastasis of enterovirus 71 (EV71) from the intestine to the whole body. EV71 is one of the main causes of hand-foot-and-mouth disease (HFMD), and its infection relies on the interaction of the canyon region of its virion surface and the glycosylation of the SCARB2 protein, which is the cellular receptor of EV71 infection. Dang *et al*[22] found that acarbose not only inhibited cellular receptors of various glycosylated viruses but also competitively blocked the canyon region of the EV71 virion surface, blocking the metastasis of EV71 from the intestine. Angiotensin converting enzyme II (ACE2) is a SARS-CoV-2 cell entry receptor[23], and glycosylation sites play an important role in

the combination of SARS-CoV-2 and its receptor[24,25]. Chloroquine was reported to block SARS-CoV-2 infection by interfering with the glycosylation of cellular receptors [26]. As previously stated, acarbose inhibited the glycosylation of EV71 receptors; additionally, patients with diabetes with COVID-19 who were treated with acarbose had better outcomes than patients who were not treated, suggesting that acarbose could improve the prognosis of COVID-19 infection by inhibiting the glycosylation of ACE2. In addition, compared to the non-acarbose group, the acarbose group had lower WBC, NLR, and CRP levels, indicating a decreased inflammatory response and further supporting the anti-SARS-CoV-2 function of acarbose. Furthermore, a previous study showed that acarbose could change the gut microbiota and then beneficially regulate the body's immune function[27]. A recent study revealed that fetal microbiome changes occurred in patients with COVID-19, characterized by depletion of beneficial commensals and enrichment of opportunistic pathogens[28]. Therefore, we inferred that acarbose might increase the baseline abundance of microbiota that had inversely correlated with COVID-19.

As previous studies reported that metformin has multiple additional health benefits in patients with diabetes[29], we anticipated that metformin would improve prognosis after COVID-19 infection; however, the results were unexpected. Scanning the literature, we found that metformin improves ACE2 stability through AMPK[30], which means that metformin may increase ACE2 availability. In addition, the median level of FPG was higher in metformin users than in nonusers, as a previous study reported that improving glycemic control substantially reduced the risk of mortality from COVID-19.

The study has some limitations. First, due to the retrospective and multiple-center study design, some information, such as patients' exposure history, the chronic disease severity and medication, diabetes medication, glycemic control and several laboratory items, was not available for all patients. There could be assay variability in different centers. Second, samples were only from Hubei Province, China; thus, more studies in other regions, even other countries, might obtain more comprehensive insight into COVID-19. However, this study is one of the largest retrospective and multicenter studies among patients with COVID-19. Additionally, this study is one of the first to investigate the influence of diabetes medications in patients with diabetes with COVID-19. The relatively abundant clinical data and numerous events also strengthen the results. The conclusion will help clinicians identify high-risk patients and choose suitable diabetes medication for patients with diabetes.

CONCLUSION

In conclusion, patients with diabetes had worse outcomes when suffering from COVID-19; however, the outcome was not related to diabetes itself but to comorbidities such as hypertension, CKD and CVD. Furthermore, the administration of acarbose could reduce the risk of death, ARDS, and shock in patients with diabetes.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) has become an ongoing pandemic and has caused considerable mortality worldwide. Previous studies have demonstrated that patients with diabetes have a higher risk of mortality from COVID-19, as well as a greater risk of developing more severe cases.

Research motivation

Diabetes was a risk factor for the progression and prognosis of COVID-19, however, the effects of diabetes or anti-diabetic medication on the mortality of COVID-19 have not been well described.

Research objectives

We aim to investigate the outcome of different statuses (with or without comorbidity) and anti-diabetic medication use before admission of diabetic after COVID-19.

Research methods

The clinical characteristics of 1422 consecutive hospitalized patients were collected. The statistical analyses were conducted with SPSS (version 25.0).

Research results

The overall survival rate was significantly lower in the diabetes group (log-rank $P < 0.01$), but the results for all-cause mortality were similar in the non-comorbidity group and diabetes only group (log-rank $P = 0.59$). Male sex [hazard ratio (HR) 2.59, $P < 0.001$], hypertension (HR 1.75, $P = 0.006$), chronic kidney disease (CKD) (HR 4.55, $P < 0.001$), cerebrovascular disease (CVD) (HR 2.35, $P = 0.006$), and age were independent risk factors for the COVID-19 mortality in multivariable Cox regression. However, diabetes alone was not an independent risk factor for mortality in patients with COVID-19.

Research conclusions

Although diabetes is associated with a higher risk of mortality in patients with COVID-19, the outcome was not related to diabetes itself. Age, hypertension, CKD and CVD were the independent risk factor of mortality.

Research perspectives

The present study calls more attention to the impact of older age and comorbid chronic disease, such as hypertension, CKD and CVD on disease progression among diabetic patients with COVID-19.

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LETTER TO THE EDITOR

Non-alcoholic fatty liver disease, diabetes medications and blood pressure

Ioannis Ilias, Costas Thomopoulos

ORCID number: Ioannis Ilias 0000-0001-5718-7441; Costas Thomopoulos 0000-0002-8491-6029.

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Ioannis Ilias, Department of Endocrinology, Diabetes and Metabolism, Elena Venizelou Hospital, Athens 11521, Greece

Costas Thomopoulos, Department of Cardiology, Elena Venizelou Hospital, Athens 11521, Greece

Corresponding author: Ioannis Ilias, MD, PhD, Consultant Physician-Scientist, Department of Endocrinology, Diabetes and Metabolism, Elena Venizelou Hospital, 2, El Venizelou Square, Athens 11521, Greece. iiliasmd@yahoo.com

Abstract

New glucose-lowering agents reduce liver enzyme levels and blood pressure (BP). Whether this finding can be extended to non-alcoholic fatty liver disease (NAFLD) patients, in whom a bidirectional association of NAFLD measures and BP has been also demonstrated, remains by and large unknown.

Key Words: Antidiabetic drugs; Blood pressure reduction; Non-alcoholic fatty liver disease; Sodium glucose cotransporter 2; Alanine aminotransferase; Aspartate aminotransferase

Core Tip: All new glucose-lowering agents reduce liver enzyme levels. Additionally, sodium glucose cotransporter 2 inhibitors can reduce both systolic and diastolic blood pressure (BP) by 3.5/1 mmHg, respectively, while glucagon-like peptide-1 agonist treatment was accompanied by systolic BP reduction of 1 mmHg. Whether this previous finding can be extended to non-alcoholic fatty liver disease (NAFLD) patients, in whom a bidirectional association of NAFLD measures and BP has been also demonstrated, remains by and large unknown.

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TO THE EDITOR

We read with interest the meta-analysis by Fu *et al*[1], which aimed to investigate the changes from baseline of selective liver enzymes, namely alanine aminotransferase and/or aspartate aminotransferase, in patients with non-alcoholic fatty liver disease (NAFLD). Patients were treated with either new glucose-lowering agents [*i.e.*, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 receptor (GLP-1) agonists, and sodium glucose cotransporter 2 (SGLT2) inhibitors] or placebo/other glucose-lowering drugs. Secondary outcomes along with the same comparison were changes from baseline of (1) different measures of body adiposity partly estimated by liver magnetic resonance, and (2) glycated hemoglobin levels. The authors clearly showed[1] that all new glucose-lowering agents reduced liver enzyme levels, whereas measures of body adiposity including body fat composition were at least numerically reduced in all cases. It would be interesting to know the changes of fatty liver index[2-4], which is a more integrated measure of liver damage in NAFLD, and whether new glucose-lowering agents can effectively reduce blood pressure (BP) levels in this pool of studies. The effect of new glucose-lowering agents against placebo on BP levels has been investigated in a pool of outcome trials[5], suggesting that among these agents, only SGLT2 inhibitors can reduce both systolic and diastolic BP by 3.5/1 mmHg, respectively, while GLP-1 agonist treatment was accompanied by systolic BP reduction of 1 mmHg. Whether this previous finding[5] can be extended to NAFLD patients, in whom a bidirectional association of NAFLD measures and BP has been also demonstrated[6], remains by and large unknown.

Beyond the above clinical considerations, we would like to emphasize on some technical issues regarding the meta-analysis by Fu *et al*[1]. First, the authors estimated changes from baseline and not differences after the intervention. Differences from baseline can bias the results in two ways, (1) because of Wilder's principle[7], indicating that reductions are higher from higher baseline levels, and (2) because in randomized studies with a limited number of participants, the levels of a given measure are not identical between treatment arms[8]. Second, another source of bias is the inclusion of placebo-controlled and active-controlled studies[9]. Although placebo is a fair comparator in this type of investigation, active-controls may have reduced the net outcome effect of new glucose-lowering agents. Third, wandering between statistical models (*i.e.*, fixed-effect *vs* random-effects) is not advised in clinical meta-analyses and a random-effects model, when gathering studies from the literature, should always - a priori - be selected irrespectively of the underlying heterogeneity [10].

The study by Fu *et al*[1] is clinically important and suggests that new glucose-lowering agents contribute to a reduction of NAFLD severity, which may partially explain the cardioprotective effect of these drugs on major outcomes[5,11].

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