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EDITORIAL

## Diabetes and data in many forms

G. R. Sridhar<sup>1</sup>

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Traditionally, capture, analysis, and usage of data were difficult. Information technology has altered the equation between capture and usability: generation of data exploded; constraints exist in analysis and appropriate use.

The rich source of clinical and biochemical data in diabetes was usually recorded on paper. It was efficient to document, but laborious to retrieve and analyze. Availability of information technology revolutionized all these. The use of electronic medical records (EMR) makes it possible to capture biographical, clinical, and biochemical data at one point and on follow-up [1, 2].

In its early years, biological science was descriptive: observing and classifying different parts of the biological system. The Human Genome Project provided a path for the ultimate in a reductionist approach: defining the units of information in terms of the nucleotide sequences which code for biological effects [3]. It is now widely accepted that information science and molecular biology have shared concepts.

To bring information technology into clinical context is beset with difficulties. Primarily, it depends on the purpose for which the data would be used (clinical care, clinical focus on a narrow area, clinical audit—although these are not mutually exclusive). Ultimately, there is a tradeoff between the depth of information that is gathered and the time that is available for each subject. This is a crucial aspect in converting paper-based record system to electronic medical records, as

the advantages of the latter are many. Good software is given, but the bottleneck in converting to EMR is “process design, understanding and supporting workflows, and the economic and social aspects of organizational change.”

Once the system is in place, advantages are obvious: structured entry of information, near-instantaneous recall, and built in rules for diagnosis and advice [1, 2].

The data can be utilized in ways other than for the purpose it was originally obtained. For instance, it could be subjected to predictive modeling using neural network methods. In the current issue, Duggal et al. used machine learning methods to predict readmission of patients who were discharged from a hospital [4]. From 9381 records, random forest was the optimal classifier using area under precision-recall curve to identify risk factors. Neural network methods have the ability to “learn” as larger sets of data are provided. It differs from statistical methods, which operate on the available circumscribed data. Machine-learning tools were used in other applications such as detecting adverse drug events from electronic medical records [5].

Access to mobile telephony led to development of mHealth apps. In this issue of the journal, Jha et al. [6] compared the effectiveness of electronic health and mobile health platform with conventional care in diabetes. A pilot study from a tertiary care hospital showed a reduction in HbA1c. Diabetes knowledge and quality of life were better in the group managed by electronic health and mobile health record.

The World Health Organization published a document on mHealth strategy [7]. eHealth is defined as the “use of information and communication technology for health” with an objective to improve service delivery and outcomes utilizing computers, internet, and mobile phones. Advantages eHealth include promotion of healthy lifestyle and improved decisions by ensuring access to care even in remote places. Of relevance to diabetes, eHealth and mHealth can be employed in remote

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Based on the Presidential oration at the Endocrine Society of India Annual Conference held at PGIMER Chandigarh on 15 November 2014

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monitoring, health education, and continual training in self-care. Studies from India have shown mHealth is feasible and improves health outcomes in subjects with diabetes. Text messaging improved health behavior related to diabetes [8]. Similarly, a pilot study of mHealth intervention was compared to usual care in three metropolitan cities across India over a 6-month period [9]. The Gather system, built on behavioral change theories to support self-management and to improve communication with physicians, resulted in improved A1c levels, similar to an earlier study reported by Shetty et al. [10]. A systematic review on web-based remote monitoring systems in the self-management of type 2 diabetes concluded that there is complexity involved in the technology as well as in its implementation [11]. These must be addressed.

Rapid advances in technology are bringing in innovative methods of low-cost, efficient care for subjects with diabetes [12]. These have been described as affording “a more fluid, real-time, and patient-centric” treatment by way of access to education material, integration of biochemical data in improving self-care [13]. More than 1000 mobile apps exist, related to chronic diseases including diabetes. The apps related to diabetes allow logging and tracking patient data, as well as exporting the data to EMRs. A number of publications show that these apps are feasible and effective in improving clinical outcomes [14]. Work in progress consists of being able to add notes to data, tracking lab results, and employing identifiers such as bar codes [15]. These apps are expected to be certified for quality control by regulatory agencies.

There have been interesting attempts where data is analyzed to learn how built environment is related to health and well-being. Built environment refers to environments that are modified by humans, which influences their behavior leading to changes in lifestyle [16]. Urban sprawl typically found in developed western countries contributes to increasing sedentary lifestyle and obesity and diabetes.

Psychosocial measures were assessed in relation to built environment using Guttman’s smallest space analysis [17]. Relations among the psychosocial measures were accounted for by “one facet with three axial sets of variables:” (a) positive well-being and energy; (b) satisfaction, impact, and social worry and diabetes worry; and (c) anxiety and depression. Prevention methods can be devised keeping the relation of built environment and psychosocial stressors. It is of interest that the data was obtained over the course of years primarily for evaluation of various psychosocial aspects of diabetes [18, 19].

The data can be analyzed using neural network methods to predict the psychological outcome based on key clinical and biographical factors [20]. Similarly, clinical data can be analyzed to identify trends in the prevalence and other characters of diseases [21, 22].

But it is really after the publication of the human genome, in all its iterations, that biological data and information science

were recognized to be intertwined. While in the past, data is difficult to obtain and analyze, newer molecular biological approaches turned the cart around—where a lot more data were available. Challenges exist to develop statistical, mathematical, and computational methods to understand and utilize [23].

There has been continuing documentation of gene expression studies in different situations: among offspring who had one parent with type 2 diabetes [24], PPARG (Pro12Ala) polymorphisms [25], and variants of a TCF7L2 gene in the north east part of Uttar Pradesh [26]. An earlier editorial in the journal summarized aspects of such studies [27].

The avowed practical implementation of the HGP was personalization of drugs; although benefits have been hard to come by, attempts are being made to match genetic profile with the individual. A review of personalized drug use in diabetes is published in this issue [28]. Although genetic variants and metabolomics studies using omics technologies have the potential to be used as biomarkers [29], translatable results are scarce. Despite a number of common variants being associated with type 2 diabetes, they explain only a very small fraction of the disease heritability. A recent effort to improve the predictive value by studying infrequent and rare variants showed they did not have a significant role in predisposing to the disease [30]. Resultantly, the major limitation in applying diabetes genomics to clinical care is the lack of genomic findings which can be used in clinical practice [31]. The way forward seems to consist of improved understanding of genetic variant interactions with environmental factors [32]. Given the low penetrance of most alleles in type 2 diabetes and poor predictive value, methods must be devised to communicate the risks accruing from genetic factors so that beneficial health outcomes can be achieved [33]. Alongside, there is an increasing need for trained researchers at the “intersection of computer science, statistics, mathematics, and their discipline of interest” to deal with data-rich but (as yet) discovery-poor situation [34, 35].

Of the many ways in which genomic data can be analyzed, the ability to impute evolutionary pathways, as demonstrated by the phylogenetic and promoter analysis of IAP gene with diabetes in the current issue of the journal, is exciting [36]. The phylogenetic and the gene promoter analysis suggested that “regulatory elements for beta cell death caused by pancreatic amyloidosis” could lead to T2DM. This is similar to the hypothesis based on bioinformatics analysis that the enzyme butyrylcholinesterase could be related to the pathogenesis of Alzheimer’s disease and type 2 diabetes mellitus [37]. The hypothesis was followed up by an animal study in which streptozotocin-induced diabetes in albino Wistar rats showed a decline in cognitive function and an increased serum butyrylcholinesterase [38]. A series of other studies have suggested that it could be one of the mediators between type 2 diabetes mellitus and Alzheimer’s disease [39]. Of greater interest is

the potential of evaluating individuals having variant butyryl-cholinesterase proteins and their susceptibility or protection from metabolic syndrome and its associated abnormalities [40, 41].

Advances in these fields not only require conceptual and technical collaboration to devise treatable options [35] but could also involve ethical issues such as the ownership of data and providing access of genetic data to the participants, i.e., in other words, to answer the question “who owns the data [42]?“

Besides analysis of biological data, availability of other forms of data provides innovative methods to assess poverty and to understand how social interactions are related to health outcomes [43, 44].

So we are at a juncture where there is a deluge of data. Methods must be devised to collect, annotate, and understand it, rather than be overwhelmed. One can expect to deal not just with science but with society, ethics, and law. Interesting times await.

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CASE REPORT

## An unusual cause for facial nerve palsy: mucormycosis

Satvinder Singh Bakshi<sup>1,2</sup>

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**Abstract** Rhinocerebral mucormycosis is a rapidly fatal fungal infection affecting mostly uncontrolled diabetics. Multiple cranial nerves can be affected; however, palsy of the facial nerve has been rarely reported. Three uncontrolled diabetic patients presented to our outpatient department with features of rapidly progressive sinusitis and unilateral facial nerve palsy which on further evaluation was proven to be mucormycosis. Patients with uncontrolled diabetes and facial nerve palsy can have an underlying mucormycosis. This is important as early diagnosis and treatment is very prudent for a favorable treatment outcome in mucormycosis.

**Keywords** Mucormycosis · Diabetes · Facial palsy

### Introduction

The term rhinocerebral “mucormycosis” was coined by Baker [1]. It is also called as “zygomycosis” as it is most commonly caused by *Rhizopus oryzae*. It has emerged as an increasing pathogen due to the rise in the incidence of diabetes mellitus and immunosuppressive treatment. After Aspergillus and Candida, it is the most common invasive fungal infection. The most common form of presentation is rhino-orbital-cerebral mucormycosis followed by pulmonary, disseminated,

and gastrointestinal types. The typical presentation is of a poorly controlled diabetic patient with fever, facial pain and swelling, nasal congestion, eyelid swelling, similar to acute rhinosinusitis [2]. On examination, the patient may have thick nasal discharge with black necrotic areas over the turbinates and septum, but this is seen in only 40 % of cases [3].

### Case series

The patients aged from 47 to 65 years (average 56 years) and were farmers by occupation. These patients were diabetic since 15–20 years (average 18 years) but were on irregular treatment. They presented with history of fever, headache, blackish nasal discharge, nasal congestion, unilateral facial pain, and retro orbital pain of 3–5 days (average 4 days). In addition, one patient presented with decreased vision in the left eye along with altered sensorium for 1 day and an ulcer over her cheek for 3 days (Fig. 1). These patients did not give any past history of chronic rhino sinusitis, trauma, dental infection, or any nasal surgery. On examination, there was tenderness over the maxillary sinus with nasal congestion and purulent, blackish nasal discharge. Two of the patients also had black necrotic debris in the nasal cavity along with necrotic patches on the hard and soft palate. These patients also had House Brackman grade V unilateral facial nerve palsy (Fig. 1). Ophthalmologic examination in one patient revealed decreases visual acuity in one eye with slowly reacting pupils. There was no restriction of extra ocular muscles.

The CT scan findings (Fig. 2) for the patients are summarized in the attached Table 1. All the patients had urine ketone positive. They were started on insulin, intra venous fluids, and other measures to control the blood sugars and treat the ketoacidosis. They were taken up for emergency debridement of the necrotic debris. The tissue was sent for histopathology

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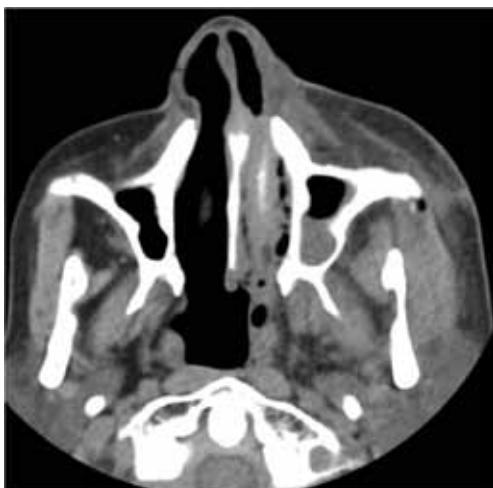
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**Fig. 1** Female patient with mucormycosis and ulcer over left cheek and facial nerve palsy (L) side

(Fig. 3) and culture and confirmed the diagnosis of mucormycosis. They were started on injecting Amphotericin B 1 mg/kg/day which was given till a cumulative dose of 1.5–2 g. They were closely monitored for signs of nephrotoxicity, hematotoxicity, and electrolyte (especially potassium) imbalance. All the patients showed improvement in facial nerve function following medical and surgical treatment, and the average time of recovery of full facial nerve function was 12 days. The patients were reassessed at 2, 4, and 6 weeks after discharge by endoscopy and were doing fine. The average duration of follow-up is 6 months at the time of writing this article



**Fig. 2** CT scan of the patient showing soft tissue opacification of the left nasal cavity with fluid collection in the left maxillary sinus and edema over L cheek

## Discussion

Mucormycosis is an opportunistic infection and can be rapidly fatal if not diagnosed and treated immediately. *R. oryzae* accounts for 60 % of all the cases and 90 % of the rhino cerebral cases. Although the disease commonly occurs in immune compromised patients especially in patients with diabetes [4], it can rarely occur in immune competent patients [3]. Other common underlying processes are metabolic acidosis, treatment with steroids or desferrioxamine, iron overload, solid organ transplant, immune deficiency syndrome, burns, and malnutrition.

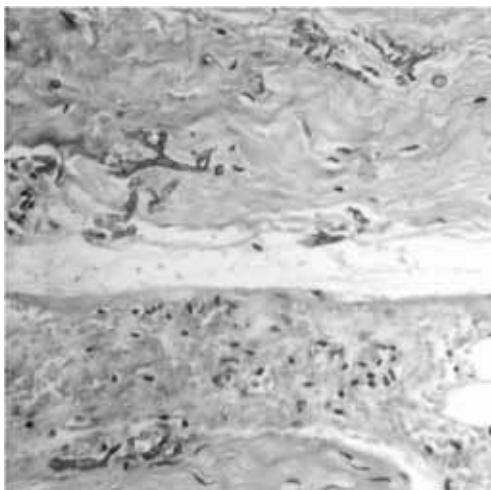
Rhino-orbital-cerebral is the most common type and is further divided into rhino maxillary, rhino orbital, and rhino cerebral types. The fungus enters the nasal passage and inoculates the nasal mucosa; from here, it spreads to the sinuses, orbit, palate and intracranially [5]. The pathogenesis is due to angioinvasion by hyphae causing thrombosis which occludes the vessels and causes ischemia [4]. This produces the characteristic black necrotic areas on which the fungus thrives and also facilitates rapid spread of the infection into the surrounding structures. Orbital invasion is common and presents as chemosis, proptosis, and ophthalmoplegia. Blindness can occur due to central retina artery occlusion or direct involvement of the optic nerve [3]. Intra cranial spread is a grave sign and carries high mortality [3].

Presentation with facial nerve palsy has rarely been reported in a few isolated case reports [6, 7]. The exact mechanism of facial nerve palsy is unknown, and no obvious pathology of the facial nerve has been identified. If the infection spreads to the infratemporal fossa via pterygopalatine fossa, it may affect the nerve as it exits the stylomastoid foramen. Another possibility is that the facial nerve palsy is incidental and may be idiopathic as in Bell's palsy. There have been case reports of mucormycosis spreading to the middle ear via the Eustachian tube, and the facial nerve can be involved in the middle ear; this was not seen in our series as all the patients had no ear complains and normal ear examination. The prognosis for return of facial nerve function has been good in our series; however, the impact of facial nerve palsy on the overall prognosis of patients with mucormycosis is yet to be determined and cannot be derived from a small case series. We suggest a systematic review of literature of larger case series of mucormycosis for determining this.

Investigations include CT scan which shows mucosal thickening with soft tissue infiltration [8]. The CT scan findings are generally non-specific and lag behind the clinical progression of the disease. The diagnosis is confirmed by either biopsy or culture of the affected tissue/secretions. Histopathological picture is of angioinvasion by irregular, broad, non-septate hyphae. The tissue can also be cultured to isolate the causative organism.

**Table 1** Clinical features, endoscopic and CT scan findings, and treatment of the mucormycosis patients with facial nerve palsy

S NO.	Age, sex	Examination findings	Endoscopy findings	CT scan findings	Treatment
1	47, M	R side purulent nasal discharge, with R cheek tenderness and numbness R grade III (HB) facial nerve palsy	Nasal congestion with edema on R side with purulent nasal discharge in R nasal cavity	Opacification with bony destruction seen in R nasal cavity and maxillary sinus	R middle meatal antrostomy and removal of edematous tissue plus Amphotericin B at 1 mg/kg/day till total cumulative dose of 1.4 g
2	63, M	L cheek insensitivity with black necrotic tissue over L inferior and middle turbinates and lateral wall Yellowish necrotic ulcer on L side of hard palate L side grade V (HB) facial nerve palsy	Black thick crusts covering left side nasal cavity lateral wall, insensitive to touch with minimal bleeding on removal	Heterogenous opacification L nasal cavity, maxillary, anterior and posterior ethmoid sinuses and fluid collection in L maxillary sinus	Nasal debridement with L complete ethmoidectomy and Middle meatal antrostomy plus Amphotericin B at 1 mg/kg/day till total cumulative dose of 1.8 g
3	58, F	L facial tenderness with 2*3 cm necrotic ulcer on the cheek and black debris on L side of nose and purulent nasal discharge L eye decreases visual acuity with mild chemosis and proptosis, no restriction in eye movements L grade V (HB) facial nerve palsy (Fig. 1)	L side purulent nasal discharge in middle meatus with black necrotic tissue covering L side of septum and inferior turbinate which does not cause pain on removing	Soft tissue mass was seen L nasal cavity with bony destruction of the lateral wall. Mucosal thickening of L maxillary, ethmoid and sphenoid sinuses. There was mild proptosis with thickening of extra ocular muscles. Soft tissue edema was also seen on the left cheek	Nasal and cheek debridement with L complete ethmoidectomy, sphenoidectomy and middle meatal antrostomy plus Amphotericin B at 1 mg/kg/day till total cumulative dose of 1.6 g



**Fig. 3** Microscopic picture showing broad, irregular, ribbon-like fungal hyphae which are aseptate with a background shows necrosis (HE, 100 $\times$ )

Surgical debridement along with medical treatment is the treatment of choice [9]. Surgical treatment includes debridement of nasal mucosa to ethmoidectomy, sphenoidectomy, medial maxillectomy to radical maxillectomy with orbital exenteration by either endoscopic or open approach. Medical treatment is with Amphotericin B 1 mg/kg/day, depending upon the patient's renal status and other underlying conditions, can give cumulative dose up to 2–3 g.

The survival rate ranges from 21 to 70 % [3] and improves with early intervention and combination of medical and surgical treatment.

## Conclusion

Mucormycosis is a rapidly progressive and fatal fungal infection, and therefore, early diagnosis and treatment is the key to survival of the patient. Patients with uncontrolled diabetes and facial nerve palsy can have underlying mucormycosis, and therefore, a thorough ENT examination is a must for all these

patients so that the patients can be managed and treated successfully.

## Compliance with ethical standards

**Conflict of interest** The author declared that he has no competing interests.

**Funding** No funding was obtained.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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CASE REPORT

# TRMA syndrome (thiamine-responsive megaloblastic anaemia): An example of rare monogenic diabetes: is thiamine a magic pill for anaemia and diabetes?

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**Abstract** Thiamine-responsive megaloblastic anaemia (TRMA) is a rare syndrome presenting with early onset non-autoimmune diabetes mellitus, megaloblastic anaemia and sensorineural deafness. We report a 16-month-old male, a youngest case of genetically confirmed TRMA syndrome in Indian phenotype, born to consanguineous parents. We found a homozygous pathogenic variant in exon 2 of the SLC19A2 gene, c.314G>A (p.Gly105Glu). The anaemia showed a good response to daily thiamine doses and was able to avoid unwanted blood transfusion. There was no benefit with regard to insulin requirement. Early detection of hearing impairment and referral to audiological treatment was possible. The report indicates that TRMA should be considered as a differential diagnosis for patients presenting with suggestive clinical symptoms which would have tremendous impact on patient management if identified early.

**Keywords** TRMA · Diabetes mellitus · Sensorineural deafness · Megaloblastic anaemia · SLC19A2 mutation

## Introduction

Thiamine-responsive megaloblastic anaemia (TRMA) (OMIM 249270) is a very rare cause of monogenic diabetes.

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It was first described by Rogers et al. [1]. It is an autosomal recessive disorder characterised by early onset diabetes mellitus (DM), megaloblastic anaemia and sensorineural deafness. Other reported clinical findings in addition to the characteristic triad include congenital heart disease, arrhythmias, retinal degeneration, optic atrophy, aminoaciduria, short stature and stroke [2].

Most of the TRMA patients originated from consanguineous families, which is consistent with autosomal recessive inheritance [3]. TRMA is caused by homozygous mutations in the SLC19A2 gene (solute carrier family 19, member 2) [3], which encodes a high-affinity thiamine transporter 1 protein (THTR-1) [4] located on chromosome 1q23.3. The human TRMA gene contains six exons that encode a protein of 497 amino acids with 12 transmembrane domains.

Its prevalence and incidence are unknown. TRMA has been reported in approximately less than 80 cases worldwide. Most of the mutations in the SLC19A2 gene lead to the production of an abnormally short, non-functional THTR-1. Other mutations change the amino acids in THTR-1, which disrupts the proper folding of the protein or prevents it from reaching the cell surface. All of these mutations prevent THTR-1 from transporting thiamine into the cell. It remains unclear how the absence of this protein leads to the seemingly unrelated symptoms of megaloblastic anaemia, diabetes and hearing loss. Research suggests that an alternative method for transporting thiamine is present in all the cells of the body, except where blood cells and insulin are formed (in the bone marrow and pancreas, respectively) and cells in the inner ear.

The disease can manifest anytime between infancy and adolescence, although often not all of the cardinal findings are manifested at onset. The variable phenotypic presentation of TRMA syndrome may cause a significant delay between the onset of symptoms and an accurate diagnosis. Treatment is symptomatic and includes pharmacological doses of thiamine

(vitamin B1) (25–75 mg/day compared to US RDA of 1.5 mg/day), which usually corrects haematological and to some extent endocrine function, but poor response regard to neurological manifestations [5]. Under high thiamine concentrations, thiamine crosses the cell membrane through passive diffusion [6]. However, the red cells remain macrocytic. The anaemia can recur when thiamine is withdrawn.

Here, we report a male patient with TRMA, belonging to a consanguineous family of South Indian origin. A homozygous mutation was found in the *SLC19A2* gene. The patient presented with the typical features TRMA, DM, SN deafness and megaloblastic anaemia.

Our case presents the youngest child with established genetic diagnosis at a very young age of 1 year, in comparison to other cases reported previously from India without genetic confirmation.

### Case presentation

A boy, 16 months of age, presented with diabetic ketoacidosis to one of the corporate hospitals. During the initial stabilisation in the PICU, he was found to be profoundly anaemic. He received blood transfusion but there was no haematological work up done for his anaemia. He was later referred to us for further diabetes management.

He was born to a second degree consanguineous couple. Parents have two more girl children who are well. In our first consultation, he was requiring insulin dose at 1 units/kg/day. Investigations revealed low C-peptide levels (0.044 ng/ml) with weakly positive GAD antibodies. He had severe vitamin D deficiency (1 ng/ml) and was given therapeutic doses of vitamin D supplements. His liver function test, TSH, vitamin B12 and serum creatinine were within normal limits. Follow up after a month showed persisting anaemia with haemoglobin 4.1 g /dl with peripheral smear suggesting of dimorphic anaemia picture. On prompting the parents, it was revealed he had speech delay and poor response to sounds. Audiological assessment showed severe to profound hearing impairment on BERA (brain evoked response audiometry) and OAE

(otoacoustic emission). On clinical suspicion of thiamine-responsive megaloblastic anaemia syndrome, he was commenced on oral thiamine supplements 75 mg once a day. After 6 weeks, he showed a significant improvement both clinical and haematological parameters (Table 1) and was also commenced on iron supplements in view of microcytic and hypochromic picture.

Considering the clinical history of the patient, mutational analysis of the *SLC19A2* gene was conducted. We detected a homozygous pathogenic variant in exon 2 of the *SLC19A2* gene, c.314G>A (p.Gly105Glu) (Fig. 1).

Following 8 months of thiamine supplements, his HbA1C was 9.2 %, there was no benefit noticed with insulin requirement and he continued to require 1 unit/kg/day.

### Discussion

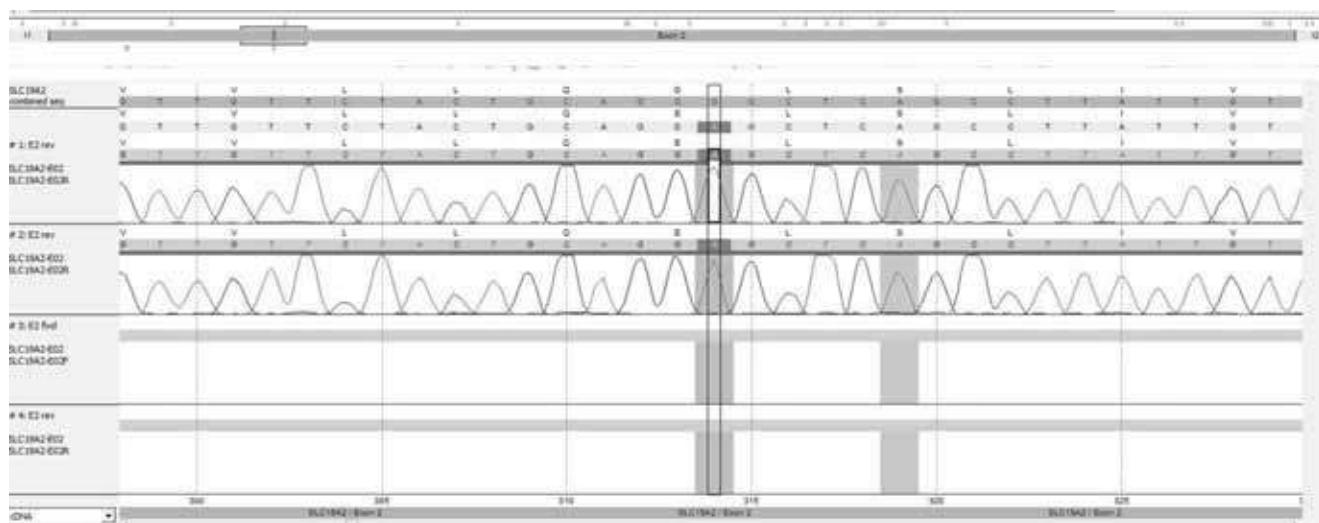
To date, this pathogenic variant is not described in the Exome Aggregation Consortium, Exome Sequencing Project or the 1000 Genomes Browser. This is the first time we detect this pathogenic variant based on Centogene's mutation/variation database (CentoMD®). It is classified in class 1 according to the recommendations of the American College of Medical Genetics. Given the result, the genetic diagnosis of thiamine-responsive megaloblastic anaemia is confirmed. In view of financial constraints, we could not carry parental carrier testing to confirm homozygosity in place of compound heterozygosity for a large deletion.

This variant has been previously detected in a homozygous state in a patient with Leber's congenital amaurosis at 10 months of age and presented in later years with hearing impairment, diabetes mellitus and megaloblastic anaemia. The variant have been described as disease-causing for thiamine-responsive megaloblastic anaemia. [7].

In our case, anaemia showed good response to thiamine and we were able to prevent this child from unnecessary blood transfusion. The hearing loss was picked up only on prompting the parents in view of clinical suspicion of

**Table 1** Improvement both in clinical and haematological parameters

Date	Hb gm/dl	WCC /mm <sup>3</sup>	Platelet lakhs/mm <sup>3</sup>	MCV Fl	MCH pg	MCHC gm/dl	Peripheral smear
ICU DKA	5.4		0.47	82	27		
1 month post-blood transfusion	4.1	10,700	0.71	94.5	29.6	31.3	Dimorphic anaemia, mild eosinophilia and thrombocytopenia Microcytic, hypochromic with good number of macrocytes and macroovalocytes
6 weeks post-thiamine supplement	11	15,900	10.1	63	25	32	Microcytic, hypochromic, eosinophilia, basophilia, thrombocytosis



**Fig. 1** Chromatogram of the patient

TRMA; otherwise, this could have been easily missed and delayed in identifying the problem.

Hearing defects may be present at an early age and in some families may even be present at birth. Progressive SN hearing loss is irreversible and may not be prevented by thiamine treatment [8]. The basis of the SN deafness is obscure and not known if it is caused by abnormalities of the cochlea or of the auditory nerve. Currently, the child is using external hearing aids and has registered for cochlear implant.

The DM is non-type I in nature. High-dose thiamine may ameliorate DM in the short term and perhaps even for decades [9]. The high level of thiamine in the pancreas is essential for its normal endocrine and exocrine function [10]. Thiamine deficiency negatively impacts insulin regulation, as the pancreatic islet cells from thiamine-deficient rats show impaired insulin secretion. This is possibly due to cell death and apoptosis.

## Conclusion

In summary, we report a genetically confirmed very young case of TRMA syndrome in a patient from a consanguineous South Indian family. The patient was found to have a homozygous pathogenic variant in exon 2 of the SLC19A2 gene, c.314G>A (p.Gly105Glu). The patient's anaemia showed a good response to daily thiamine doses. The report indicates that TRMA should be considered as a differential diagnosis for patients presenting with suggestive clinical symptoms, which would have tremendous impact on patient management if identified early.

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interpretation on our case and providing relevant information associated with TRMA.

**Author's contributions** SOS drafted the original manuscript. DV took part in the clinical care of the patient and edited the manuscript. All authors have read and approved the final version of the manuscript submitted.

## Compliance with ethical standards

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**Competing interests** The authors declare that they have no competing interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent** Written informed consent was obtained from the patient for publication of this case report.

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CASE REPORT

## Concurrent onset of type 2 diabetes mellitus and central diabetes insipidus in an adult male

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**Abstract** Polyuria is a relatively common symptom which can be caused by a wide range of conditions including uncontrolled diabetes mellitus but also the more rare central diabetes insipidus. The association of the two diagnoses in adult patients is extremely rare. We report the case of a 43-year-old male with significant polyuria and polydipsia (>10 l/24 h) developed progressively over almost a year. Six months before admission in our department, uncontrolled type 2 DM was diagnosed and polyuria was interpreted as a result of his significant glycosuria. However, despite adequate sulfonylurea treatment with normalization of glycemic levels, polyuria persisted and even worsened. Upon admission, polyuria with a very low urine specific gravity and osmolality was noted (suggestive of increased water loss). A water deprivation test was performed and confirmed central diabetes insipidus. Treatment with oral desmopressin was initiated and polyuria disappeared. The concomitant occurrence of DM and DI is extremely rare in adult patients. In such a rare situation, polyuria is usually attributed to

uncontrolled DM and concurrent DI can be easily overlooked. Low urine specific gravity and osmolality as well as persistence of polyuria despite adequate control of DM are useful clues to reach the correct diagnosis.

**Keywords** Diabetes mellitus · Polyuria · Diabetes insipidus

### Introduction

A wide range of differential diagnoses should be taken into account in patients with polyuria and polydipsia. Common causes of polyuric states include uncontrolled diabetes mellitus (DM), kidney failure, hypokalemia, hypercalcemia, but primary water homeostasis disorders [diabetes insipidus (DI), psychogenic polydipsia] need to be considered if more clinically obvious conditions are excluded from the initial evaluation [1]. Concomitant diagnosis of both diabetes insipidus and diabetes mellitus has only been rarely reported. The coexistence of the two conditions is well-known in rare genetic syndromes (Wolfram syndrome, DIDMOAD) diagnosed in childhood [2, 3]. Rarely such an association has been reported in adults with a previous long-term history of diabetes mellitus [4]. A careful laboratory assessment is needed in such a patient in order to detect the coexistence of diabetes insipidus. We present the rare case of an adult with an almost concomitant diagnosis of DI and DM.

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**Table 1** The laboratory values of our patient during admission

Parameter	Measured value	Normal values
FSH (mIU/mL)	1.49	1.27–19.26
LH (mIU/mL)	3.38	1.24–8.62
Testosterone (ng/mL)	2.06	1.75–7.81
Cortisol ( $\mu$ g/dL)	16.73	5–25
TSH ( $\mu$ IU/mL)	1.43	0.4–4.5
Free 4 (pmol/L)	12.85	9–19
Urinary volume/24 h	13 l	<2.5–3
Plasma osmolality (mosm/kg)	299	285–295
Sodium (mmol/L)	138	135–145
Urinary osmolality in 24 h-urine (mosm/kg)	100	500–1200
Urinary osmolality after dehydration (mosm/kg)	285	>600

## Case report

A 43-year-old man was referred to our department for the investigation of polydipsia and polyuria. The symptoms had occurred progressively, approximately 1 year before admission; lethargy and dizziness were initially associated. After 6 months, uncontrolled type 2 DM was diagnosed (a jeun glycemia 16.66 mmol/l, glycosuria) and treatment with sulfonylureas was initiated. Under treatment, the glycemic levels normalized, the lethargy and dizziness almost disappeared, but the polydipsia (approximately 12–13 l of fluids daily) remained uninfluenced. During the last 6 months before being admitted to our department, the patient lost 10 kg.

On admission, the patient denied any other symptoms as well as any history of head trauma. His family and personal medical history were clear (except for the recently diagnosed DM, adequately controlled with oral antidiabetics).

The clinical examination revealed a slightly overweight male, with normal general clinical examination, without any signs of hypopituitarism or hypothalamic involvement (apart from the severe polyuria 13 l/24 h).

The laboratory values (see Table 1) revealed normal pituitary function, polyuria with very low urinary osmolality, normal serum electrolytes. We performed the standard water deprivation test and confirmed the presence of central diabetes insipidus (still very low urinary osmolality after dehydration confirmed diabetes insipidus, moderate increase early after desmopressin administration, and more sustained thereafter, confirming the central origin of the condition—see Table 2).

The CT imaging of the hypothalamic-pituitary area (unfortunately, MRI examination was not available) revealed no abnormalities—see Fig. 1.

Chronic desmopressin treatment was initiated with sublingual desmopressin (Minirin melt) 120  $\mu$ g daily. The clinical response was very favorable, with

**Table 2** The laboratory values obtained during the dehydration test

Time	Body weight (kg)	Urine volume (mL)	Urine osmolality (mosm/kg)	Plasma osmolality
7	81.7	550	100	290
8	81.3	430	146	-
9	80.7	390	177	-
10	80.5	370	205	-
11	80.2	310	221	299
12	80	245	250	-
13	79.85	220	285	303
After taking this last sample, nasal desmopressin spray was administered				
14	80.2	15	368	296
17	81.1	95	578	290



**Fig. 1** Coronal CT section of the hypothalamic-pituitary area revealing no abnormalities related to symptomatology (normal-sized pituitary gland with minimal heterogeneity, thin pituitary stalk)

normalization of diuresis, thirst sensation, and urinary osmolality.

## Discussion

Central diabetes insipidus (CDI) is due to a deficiency in the hypothalamic synthesis of arginine vasopressin (antidiuretic hormone) or in the secretion of the hormone from the posterior pituitary (as opposed to nephrogenic DI, caused by resistance to arginine-vasopressin action in the kidney). The prevalence of the condition is low (1:10,000), but that of diabetes mellitus is continually growing, so after a long follow-up, a significant percentage of diabetes insipidus cases also develop diabetes mellitus [5].

The coexistence of DI and DM from the diagnosis in the same patient has been rarely reported in Wolfram syndrome (known as DIDMOAD), a congenital genetic disorder diagnosed in childhood, characterized by the association of central DI with type 1 DM, optic atrophy, deafness, and infantilism [2].

Usually, the patients with Wolfram syndrome first develop non-autoimmune, insulin-deficient DM in their childhood, followed by optic atrophy and diabetes insipidus later in their lifetime [6]. Familial disease including DI and DM without the other comorbidities has also been described [2].

The coexistence of CDI and type 2 DM has been reported in few adult patients, most of whom had a long history of type 2 DM before developing CDI [4, 7, 8]. To our knowledge, only one case with concomitant onset of both conditions in adults has been previously reported. [9] The possibility of concomitant onset

should be borne in mind because if DI is overlooked and uncontrolled DM is considered to be exclusively responsible for polyuria, serious consequences can develop in time.

CDI can be caused by a number of conditions that affect the hypothalamus or pituitary (e.g., trauma, brain tumors, autoimmune disorders, infections); however, in almost half of the cases, no such etiological factor is obvious and a diagnosis of idiopathic DI is made [10]. If further investigation in a research laboratory is performed, in many of these cases, an autoimmune etiology of the condition is likely [11]. In our case, unfortunately, MRI examination was unavailable so the possible lack of the “bright spot” (typical hyperintense appearance of the normal neurohypophysis) could not be evidenced.

In our case, the very high urine output could suggest a possible associated component of primary, psychogenic polydipsia. However, the normal value of serum sodium concentration together with the increased plasma osmolality argues against this diagnosis. Even more, the significant response to desmopressin administration, as shown during the water deprivation test, is typical for central diabetes insipidus.

Due to the extreme rarity of the concomitant onset of DM and idiopathic central DI in adults and the different etiologies of the two diseases, it is unlikely that this concomitant occurrence could be the result of a single pathogenetic chain of events. It appears more probable that their association from the time of diagnosis in our case is a coincidence, but one that underlines the absolute necessity of completely investigating polyuria and polydipsia, even in cases with uncontrolled DM. Due to the rarity of concomitant associations reported so far, it is difficult to suggest a direct pathogenetic link between the two disorders.

A very careful laboratory assessment is needed in order to diagnose diabetes insipidus in a diabetic adult. Useful initial clinical clues are the lack of glycosuria and marked hyperglycemia (characteristic of uncontrolled DM). The decisive laboratory parameter is the low urine specific gravity (characteristic of diabetes insipidus, unusual in a case with polyuria due to uncontrolled diabetes mellitus [7]).

## Conclusions

An almost concomitant diagnosis of DM and DI is extremely rare in an adult patient and most likely does not point toward a common pathogenesis. However, it is important to be acknowledged that the two conditions can coexist from the time of the diagnosis and to be careful when attributing polyuria and polydipsia solely to uncontrolled DI. If significant polyuria persists despite adequate control of DM, it is mandatory to order appropriate tests to reach a correct diagnosis.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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## Glycemic control is difficult to attain in type 2 diabetes mellitus despite insulin therapy

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The standard way of treating a type 2 diabetic patient is to start them on monotherapy with oral hypoglycemic agents (OHAs), then proceed to combination therapy, and when OHAs are no longer found to control blood glucose adequately, start them on insulin with or without stopping the OHAs in the hope that addition of insulin will achieve euglycemia [1]. But in practice, we often find that glycemic control is not optimal in majority of type 2 diabetic patients more so when patients are on insulin than when they are on OHAs [2]. There was not enough scientific data to support this view. Hence, a study aiming to assess the level of glycemic control in type 2 diabetic patients and to compare the glycemic control in type 2 diabetic patients taking insulin with or without OHA vs those on OHA alone was planned.

Adult type 2 diabetic patients who were on treatment for their diabetes started by a general practitioner at their local place for atleast 2 years were included in the study.

All these patients were coming to a tertiary referral university teaching hospital for the first time in 2010. One hundred patients taking insulin with or without OHAs and 100 on OHA alone were included in the study. The former was taken as group 1 and the latter as group 2.

Details of duration of diabetes, HbA1c, FPG, PPPG, presence of diabetic retinopathy, nephropathy and neuropathy were analyzed. The details of treatment are given in Table 1.

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The results comparing the different parameters between groups 1 and 2 are given in the Table 2.

The study revealed an overall poor control of type 2 diabetes mellitus in our community in a general practice set up, especially when they are in an advanced stage of their disease inspite of insulin therapy. Only 16 % of the total population ( 9% on insulin and 24 % on OHAs) had the target HbA1c of < 7 %. Those on insulin had longer duration of disease, higher HbA1c, higher FPG and PPPG, and higher incidence of microvascular complications. The reasons for a poor glycemic control in those on insulin with or without OHA compared to OHA alone may be manifold.

- 1) As duration of diabetes increases, the endogenous insulin reserve decreases, and hence, more people will need exogenous insulin, and it is well known that glycemic control worsens when endogenous insulin reserve decreases. Thus, those on insulin have a more severe form of disease and hence have a poor glycemic control.
- 2) It is possible that insulin treatment increases the body weight and hence worsens the glycemic control.
- 3) Once patients are started on insulin, both the physician and the patient will be lax in their attitude towards diabetes thinking that insulin is anyway going to control the hyperglycemia.
- 4) Frequent monitoring of blood glucose is required when patients are on insulin which probably is not practically being done resulting in uncontrolled hyperglycemia.
- 5) The pharmacokinetic profile of the available insulins is far from ideal and may be contributing to the poor control.

The fact that HbA1c correlated with duration of diabetes will support the first reason for this poor control on patients on

**Table 1** Details of treatment of groups 1 and 2

Medicine	Premixed insulin	Basal insulin	Basal bolus insulin	Sulfonyl urea	Metformin	Pioglitazone	Others	Combination
Group 1 (%)	76	20	4	40	50	20	6	85
Group 2 (%)	–	–	–	86	77	15	8	82

insulin and OHA. The level of glycemic control in our population is worse when compared to developed countries like US where more than 50 % of type 2 diabetic patients achieve an HbA1c of <7 [3]. This is going to increase the burden of microvascular complications in the future in a developing country like India.

**Table 2** Comparison of groups 1 and 2

Variable	Insulin +/−OHA Group 1	OHA Group 2	P value
HbA1c	9.5 ± 1.9	8.4 ± 1.8	<0.001
FPG mg/dl	171 ± 70	160 ± 57	0.208
PPPG mg/dl	267 ± 89	252 ± 96	0.253
Diabetic retinopathy (%)	41 %	11 %	<0.001
Diabetic nephropathy (%)	45 %	33 %	0.082
Diabetic neuropathy (%)	60 %	46 %	0.047
HbA1c ≤ 7	9 %	24 %	0.004
FPG ≤ 130 mg%	30 %	33 %	0.648
PPPG ≤ 180 mg%	16 %	23 %	0.212
Duration of diabetes	13 ± 7.7 years	8 ± 5.1 years	<0.001

To conclude, this study assessing the glycemic control of type 2 diabetic patients in a general practice set-up shows that good glycemic control was attained only by a dismal 16 % of patients. On comparing those treated with OHA alone and insulin with or without OHA, the patients on insulin have a much worse glycemic control compared to those on OHA. More stringent measures need to be undertaken to improve the glycemic control of type 2 diabetic patients especially when they reach the insulin-dependent stage.

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LETTER TO THE EDITOR

## Non-invasive blood glucose monitoring is an elusive goose

Devi Dayal<sup>1</sup>

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Sir,

The recent article by Chowdhury MK et al. made for an interesting read [1]. The authors showed that their indigenously developed ‘modulated ultrasound and infrared light’ technique can be used for non-invasive blood glucose (NIBG) measurement with reasonable accuracy. While their conclusions based on the performance in 60 adults appear promising, the practical utility of their system will only be known after it is tested on larger numbers of patients.

Diabetes is one of the fastest growing diseases and is expected to affect 366 million people in 2030 [2]. In order to maintain relatively normal lives, people with diabetes need frequent measurements of blood glucose. This is routinely done by an invasive method that involves needle prick and may hurt or cause bruising. Many people with diabetes particularly children have needle phobia and may simply refuse the testing. Additionally, social unacceptability of blood droplets and concerns about blood-borne diseases with the repeated needle pricks is reported. Thus, a reliable system of glucose monitoring that is painless and does not involve blood would be desirable for people with diabetes and will give them a freedom of monitoring their blood glucose as often as required [3]. It is obvious that such a device will become an instant medical and commercial success capable of improving the lives of millions of diabetics forever.

The world of NIBG monitoring, however, is replete with failures. Over a hundred approaches (most commonly spectroscopic and optical rotation techniques) have been tried till now without much clinical success [3]. Every reported technique has vanished after creating the initial hype [3]. Although the number of patents filed for NIBG has increased exponentially over the past four decades, none has yielded a commercially successful device [3]. Therefore, the scepticism about every new report is natural. In particular, the use of infrared spectroscopy reported in the present study has several limitations such as absorption by other biological chromophores, interferences due to stratum corneum resulting in poor signal to noise ratio, calibration issues, baseline drift, thermal noise, physiological and environmental factors and wavelength selection issues that need to be addressed in future studies [4]. The authors themselves noticed a number of interferences and suggested measures to be taken in future studies to reduce their effect on results [1]. Also, the NIBG measurements at the extremes of blood glucose values should have been included to allow the assessment of system’s performance at most critical times. Calculation of the constant error could have further added to the clinical utility of the system.

Thus, NIBG has so far been an elusive goose or the deceitful turkey which has defeated its most determined hunters [3]. It is heartening to know that Indian scientists have ventured into an area dominated by scientists from the developed countries. The authors must continue their pursuit to achieve the desired goal.

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### Response from the authors

First, thank you for your valuable time to read our research article with such an interest and motivation.

We are also in the way of conducting massive clinical trials for the establishment of our noninvasive technique.

However, prior to massive clinical trials, at present our research group is working over determining blood glucose levels at the extreme ranges with an acceptable clinical significance. We hope that within one or two years, we will be able to do that. The suggestion provided by the respected reader is valuable and appreciating. Most of the interference factors are already in our path of investigations. However, we will incorporate all other issues as pointed by the respected reader in our future work.

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# Association between SNPs in *AdipoQ*, +45 T>G and +276 G>T, and adiponectin levels in the Korean Chinese population in Yanbian, China

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**Abstract** This study investigated the association between single nucleotide polymorphisms (SNPs) in *AdipoQ*, +45 T>G and +276 G>T, and adiponectin levels in the Korean Chinese population in Yanbian, China. A total of 329 subjects were involved in this study, including 178 female and 151 male individuals. All of them are ethnic Koreans living in Yanbian, aged from 31 to 70, and 58 % of them were diagnosed with type 2 diabetes (T2D). Items tested and calculated include total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG), body fat percentage (BF%), fasting plasma insulin (FPI), plasma adiponectin (PA), and homeostasis model assessment of insulin resistance (HOMA-IR). SNPs were screened with the method of SNaPshot.  $P<0.05$  was defined as the statistically significant threshold. Results include the following: (1) PA levels in the T2D group are much lower than those in the normal group ( $P<0.001$ ); (2) the distribution of genotypes of SNPs +45 T>G and +276 G>T was determined to be in Hardy-Weinberg equilibrium ( $P>0.05$ ) and complete linkage disequilibrium ( $|D'|=1.0$ ); (3) PA levels in females are much higher than those in males no matter which group they belong

to (normal or T2D group) ( $P=0.004$  and  $P=0.018$ ); (4) PA levels are higher in the individuals with +45G as a dominant allele than those in individuals with homozygous genotype TT at locus +45 in the normal group ( $P=0.037$ ); and (5) of SNPs +45 T>G and +276 G>T, no risk factor of genotype or allele was found ( $P>0.05$ ). Three conclusions can be drawn: (1) PA levels are lower in T2D patients than in normal persons; (2) PA levels are higher in females than in males; (3) PA levels are higher for normal individuals with +45G allele than those with homozygous TT genotype at locus +45 in *AdipoQ*.

**Keywords** *AdipoQ* · Adiponectin · Hypoadiponectinemia · Type 2 diabetes

## Introduction

It had been demonstrated in previous studies that hypoadiponectinemia is an independent risk factor responsible for the development of type 2 diabetes (T2D) [1–4]. Single nucleotide polymorphism (SNP) +45 T>G (rs2241766) is located in exon 2 of *AdipoQ*, participates in coding of the 15th amino acid of adiponectin, and caused a synonymous mutation (Gly to Gly). Its polymorphisms were reported to be related to adiponectin levels in Chinese and Japanese populations and related to T2D in Quebec families [5]. Furthermore, Li et al. reported an increased risk of T2D for the +45G allele in the Han Chinese population in Yunnan, China [6], while Wang et al. reported no association between SNP +45 T>G and T2D in Han and Yi Chinese populations in Sichuan, China [7]. For another SNP in intron 2 of *AdipoQ*, +276 G>T (rs1501299), its polymorphisms were reported to be related to

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adiponectin levels in European Caucasians and related to T2D in German, Italian, and Japanese populations [5], but its association with T2D was not found in the Han Chinese population [6]. Considering that the association between SNPs +45 T>G and +276 G>T and adiponectin levels and even T2D might be race-specific, to make sure if there are influences of the SNPs on adiponectin levels, it is necessary to investigate more racial cohorts. In this study, we tested the association between the two SNPs in *AdipoQ* and adiponectin levels in the Korean Chinese population in Yanbian, China.

Yanbian was part of Manchuria located contemporarily on the northeast of China and bordered with North Korea. Manchuria was once separated by Manchurians who were the administrators of the Qing Dynasty, for political and military purposes, as a reversed land in case the Han Chinese regained control over China. However, this policy had finally been abandoned in the late Qing Dynasty because of the enormous benefits in agriculture, forestry, and business in this region and the attempt of Russian encroachment. The first wave of Koreans migrated to Yanbian across the border from the Korean peninsula at this time. Later on, Korean immigrants successively moved to Yanbian for financial or political reasons. Their descendants were born in China and possessed Chinese nationalities (so-called Korean Chinese). Since recently the economic growth in Yanbian is relatively slow, some of them choose to go to South Korea or big cities in China. Therefore, the number of Korean Chinese in Yanbian is shrinking. So far, as a Korean Autonomous Prefecture, Yanbian has 2,271,600 permanent residents composed of many races; of them, 39 % are Korean Chinese (Sixth National Population Census of the People's Republic of China, 2010).

## Subjects and methods

### Subjects

A total of 329 subjects were involved in the study, including 178 female and 151 male individuals. All of them are pure ethnic Koreans (traced back to three generations) with Chinese nationalities living in Yanbian from birth, and aged from 31 to 70 years. Fifty-eight percent of them were diagnosed with T2D.

### Clinical data measurements

Total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) were measured on the 7600 Clinical Analyzer (Hitachi, Ltd., Chiyoda, Tokyo, Japan) by Yanbian University Hospital (Yanji, Jilin, China). Body mass index (BMI) was calculated with the

formula  $BMI = \text{weight}/(\text{height})^2$ . Body fat percentage (BF%) was measured with the InnerScan Body Composition Monitor (Tanita Corporation, Itabashi-Ku, Tokyo, Japan). Fasting plasma insulin (FPI) and plasma adiponectin (PA) were measured with the Human Insulin ELISA Development Kit (PeproTech, Rocky Hill, NJ, USA) and the Human Adiponectin/Acrp30 Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) by Shanghai Westang Bio-Tech Co., Ltd. (Shanghai, China). Insulin resistance was estimated with the homeostasis model assessment of insulin resistance (HOMA-IR) which is calculated with the formula  $HOMA-IR = FPG \cdot FPI / 22.5$ .

### Extraction of genomic DNA

Genomic DNA was extracted with the AxyPrep Blood Genomic DNA Miniprep Kit (Corning Life Sciences—Axygen Inc., Union City, CA, USA).

### PCR amplification

PCR was performed on Techne TC-412 (Bibby Scientific Ltd., Stone, Staffordshire, UK) with GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA). Primers are F: 5'-AGAGGCACCATCTACACTCATC-3' and R: 5'-GTCCTTGTAGGTCCCACTG-3' synthesized by Beijing AuGCT Biotechnology Co., Ltd. (Beijing, China). Amplification was conducted at an annealing temperature of 56–53 °C, obtaining a product of 478-bp DNA segments.

**Table 1** Clinical data

Item	Normal group	T2D group	Reference range	P value
n (F/M)	138 (73/65)	191 (105/86)		0.709
Age (years)	59.25±9.35	59.42±7.83		0.844
BMI ( $\text{kg}/\text{m}^2$ )	23.52±3.82	25.26±3.66	18.5–23.9	<0.001
BF% (%)	26.29±6.82	29.11±7.87	25.0–28.0	0.002
FPG (mmol/L)	5.44±1.57	8.97±4.13	3.90–6.10	<0.001
TC (mmol/L)	4.99±0.99	5.20±1.08	3.90–5.90	0.072
TG (mmol/L)	1.92±1.92	2.37±1.64	0.23–1.78	0.017
HDL-C (mmol/L)	1.65±0.48	1.39±0.36	1.23–4.50	<0.001
LDL-C (mmol/L)	2.86±0.76	2.75±0.70	2.82–3.65	0.230
PA ( $\mu\text{g}/\text{mL}$ )	6.86±6.70	3.07±2.69		<0.001
FPI (ng/mL)	24.92±26.29	23.82±37.13		0.752
HOMA-IR	5.81±10.81	9.97±11.52		0.012

Data is expressed as mean±SD. P values were calculated with  $\chi^2$  and t tests. Reference ranges are adopted by Yanbian University Hospital for defining normal values

**Table 2** Hardy-Weinberg equilibrium

Normal group				T2D group			
+45 T>G				+45 T>G			
TT	TG	GG	P value	TT	TG	GG	P value
66	63	9	0.496	97	81	13	0.776
+276 G>T				+276 G>T			
GG	GT	TT	P value	GG	GT	TT	P value
69	59	10	0.862	93	79	19	0.934

P values were calculated for Hardy-Weinberg equilibrium.

### SNP screening

SNPs were screened with the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Inc., Foster City, CA, USA). Probes are ttttttttttttttttttttttttttttGCTATTAGCTCTGCCGG for screening SNP +45 T>G and ttttttttttttttttttttCTAGGCCT-TAGTTAATAATGAATG for screening SNP +276 G>T.

### Statistical analysis

All clinical data were kept with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Their means and standard deviations (SDs),  $\chi^2$  test, *t* test, and one-way ANOVA were calculated with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Hardy-Weinberg equilibrium was determined with the Hardy-Weinberg equilibrium calculator (<http://www.oege.org/software/hardy-weinberg.html>) [8]. Linkage disequilibrium was determined with CubeX (<http://www.oege.org/software/cubex/>) [9].  $P<0.05$  was defined as the statistically significant threshold.

## Results

### Clinical data

Subjects were divided into two groups: one was composed of individuals who were diagnosed with T2D based on the criteria of diagnosis of diabetes [10], called the T2D group, and the other is composed of all normal non-diabetic persons, called the normal group. Clinical items, values of these items in the normal and T2D groups, reference ranges, and *P* values are shown in Table 1. In addition to BMI, BF%, FPG, TG,

HDL-C, and HOMA-IR, PA levels are also quite different between the two groups; PA levels in the T2D group are much lower than those in the normal group ( $P<0.001$ ).

### Hardy-Weinberg equilibrium and linkage disequilibrium

Frequencies of genotypes of SNPs +45 T>G and +276 G>T were determined in Table 2; all *P* values are greater than 0.05, so the distributions can be considered in Hardy-Weinberg equilibrium. Therefore, the subjects involved in this study can be representative of the whole population of Korean Chinese in Yanbian, China. For linkage disequilibrium,  $|D'|s$  in the normal and T2D groups are all equal to 1.0, so the loci can be considered in complete linkage disequilibrium.

### PA levels with genders

To make sure that the PA levels for different genders are significantly different, mean PA levels of females and males in the two groups were calculated (Table 3). This table shows that PA levels are significantly different with genders. PA levels in females are much higher than those in males no matter which group they belong to (normal or T2D group) ( $P=0.004$  and  $P=0.018$ ).

### PA levels with genotypes

PA levels were tested with genotypes in the normal and T2D groups based on additive, dominant, and recessive models, respectively (Table 4). Influences of genders on PA levels can be ignored here since the female/male ratios among genotype-defined subgroups have no significant differences (all *P* values are greater than 0.05). Based on the table, PA levels are higher in individuals with +45G as a dominant allele than those in individuals with homozygous genotype TT at locus +45 in the normal group ( $P=0.037$ ). But this phenomenon did not appear in the T2D group.

### Distribution of genotypes and alleles

To examine if the genotypes and alleles of SNPs +45 T>G and +276 G>T are directly associated with T2D development, the frequencies of these genotypes and alleles had been calculated and the differences between normal and T2D groups had been compared with the  $\chi^2$  test (Table 5), but no significant

**Table 3** PA levels with genders

	Normal group			T2D group		
	Female	Male	P value	Female	Male	P value
n	73	65		105	86	
PA (μg/mL)	8.28±7.71	5.10±4.66	0.004	3.53±3.07	2.61±2.16	0.018

Data is expressed as mean±SD. *P* values were calculated with the *t* test

**Table 4** PA levels with genotypes

	Normal group			T2D group			<i>P</i> value
	+45 T>G	+276 G>T	+45 T>G	+276 G>T	TG	GG	
Additive model	TT	TG	GG	<i>P</i> value	GG	TT	
<i>n</i> (F/M)	66 (37/29)	63 (32/31)	9 (4/5)	0.728	69 (39/30)	59 (27/32)	0.254
PA ( $\mu\text{g/mL}$ )	5.66±4.68	8.27±8.16	5.99±6.81	0.068	7.05±7.14	6.55±6.24	7.49±6.87
Dominant model	TT	TG+GG	<i>P</i> value	GG	GT+TT	TT	3.02±2.67
<i>n</i> (F/M)	66 (37/29)	72 (36/36)	0.476	69 (39/30)	69 (34/35)	0.394	97 (54/43)
PA ( $\mu\text{g/mL}$ )	5.66±4.68	7.97±8.00	0.037	7.05±7.14	6.70±6.31	0.752	94 (51/43)
Recessive model	TT+TG	GG	<i>P</i> value	GG+GT	TT	<i>P</i> value	3.02±2.67
<i>n</i> (F/M)	129 (69/60)	9 (4/5)	0.599	128 (66/62)	10 (7/3)	0.261	178 (99/79)
PA ( $\mu\text{g/mL}$ )	6.93±6.71	5.99±6.81	0.670	6.81±6.71	7.49±6.87	0.737	3.08±2.68

Data is expressed as mean±SD. *P* values were calculated with the  $\chi^2$  test, one-way ANOVA, and *t* test.

**Table 5** Distribution of genotypes and alleles

	+45 T>G			+276 G>T			<i>P</i> value	T2D group	<i>P</i> value
	Normal group			T2D group					
Additive model	TT	TG	GG	TT	TG	GG	<i>G</i>	<i>T</i>	<i>T</i>
<i>n</i> (%)	66 (47.8)	63 (45.7)	9 (6.5)	97 (50.8)	81 (42.4)	13 (6.8)	0.842	69 (50.0)	59 (42.8)
Dominant model	TT	TG+GG	<i>T</i>	TT	TG+GG	<i>G</i>	<i>G</i>	GT+TT	GG
<i>n</i> (%)	66 (47.8)	72 (52.2)	97 (50.8)	94 (49.2)	94 (49.2)	0.596	69 (50.0)	69 (50.0)	93 (48.7)
Recessive model	TT+TG	GG	<i>T</i>	TT+TG	GG	<i>G</i>	GG+GT	TT	GG+GT
<i>n</i> (%)	129 (93.5)	9 (6.5)	T	178 (93.2)	13 (6.8)	0.919	128 (92.8)	10 (7.2)	172 (90.1)
Allele	T	G	<i>G</i>	G	T	<i>G</i>	T	G	T
<i>n</i> (%)	195 (70.7)	81 (29.3)	275 (72.0)	107 (28.0)	0.708	197 (71.4)	79 (28.6)	265 (69.4)	117 (30.6)

Data is expressed as frequency and its percentage is in parentheses. *P* values were calculated with the  $\chi^2$  test

difference was found. This result indicates that the SNPs +45 T>G and +276 G>T are not the direct or unique risk factors for T2D development.

## Conclusions

According to the results above, three conclusions for the Korean Chinese population in Yanbian, China, can be drawn: (1) PA levels are lower in T2D patients than in normal persons; (2) PA levels are higher in females than in males; and (3) PA levels are higher for those normal individuals with the +45G allele than those with the homozygous TT genotype at locus +45 in *AdipoQ*.

## Discussion

In this study, we investigated 329 Korean Chinese individuals in Yanbian, China, mainly in trying to find the association between SNPs in *AdipoQ*, +45 T>G and +276 G>T, and adiponectin levels. Summarizing the results of the study, we can get three outcomes about adiponectin levels expressed in this population.

First, adiponectin levels are lower in type 2 diabetics. This finding is consistent with a series of previous studies [4, 11–15]. It had been demonstrated that adiponectin has the function of upregulating glucose transporter 4 (GLUT4) translocation to the cell membrane by stimulating the interaction between the adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif 1 (APPL1) and a small GTPase Rab5 [16, 17]. This study on Korean Chinese is hoped to provide additional evidence on a new race to the theory.

Second, adiponectin levels are higher in female individuals. This result is consistent with Li et al.'s research on original Chinese [18], but inconsistent with Tsai et al.'s research on Taiwanese, which suggests that there is no significant difference in adiponectin levels between genders [19]. The mechanism of sexual effects is still unclear. It may vary with racial specificities. Further studies will be necessary to test the hypothesis and demonstrating the mechanism.

Third, for normal individuals in the population, adiponectin levels are higher for those with allele G at locus +45 in *AdipoQ* compared to the ones with homozygous TT genotype. This phenomenon is consistent with Mackevics et al.'s research on German Caucasians [20]. Considering together with the conclusion above that adiponectin levels are lower in T2D patients, allele +45G may be a protective factor for preventing T2D development. However, we did not get the same result in the T2D group. This different outcome may be attributed to the fact that decreased adiponectin levels in type 2 diabetic patients narrowed the gap of PA levels between subjects with

TT homozygous genotype at locus +45 and their counterparts with at least one G allele, leading to no significant difference being observed. For SNP +276 G>T, we did not find any significant difference in adiponectin levels between genotype-defined or allele-defined subgroups no matter which group they belong to (normal or T2D group). But several previous studies did in other populations, e.g., Hara et al.'s research on the Japanese [21], Menzaghi et al.'s research on Italian Caucasians [22], and Mackevics et al.'s research on German Caucasians [20]. This deviation may suggest that the influence of SNP +276 G>T on the adiponectin expression may be diverse for different races.

Furthermore, we did not find a direct association between any genotype or allele of the two SNPs and T2D development. This result is partially consistent with Li et al.'s research on the Han Chinese [6] and completely inconsistent with Hara et al.'s research on the Japanese population [21]. Given that hypoadiponectinemia may not be the direct or unique cause of T2D development and many factors may affect the process, race-specific factors that act in human metabolism may have an influence on adiponectin expression or degradation. More evidences studied on different races and more researches focusing on the mechanism of adiponectin actions are pressingly needed.

Limited by time and funds, we confined our research on only mass genetics. Hence, this study would have limitations on the scope of the molecular mechanisms of adiponectin in the population we studied. And we are trying our best to spread our research further in the future.

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**Conflict of interest** There is no conflict of interest.

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# Gene expression profiling of the peripheral blood mononuclear cells of offspring of one type 2 diabetic parent

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**Abstract** Several lines of evidence from studies of both twins and offspring of people with type 2 diabetes have shown the importance of genetics in its pathogenesis. Impaired glucose tolerance (IGT) may reflect these genetic changes during the prediabetic stage. Thus, we performed a comprehensive analysis of the gene expression profiles of the peripheral blood mononuclear cells among offspring of one type 2 diabetic parent with normal glucose tolerance and impaired glucose tolerance in comparison to newly diagnosed diabetics and normal controls. Data were analysed from offspring of one type 2 diabetic parent. Gene expression profiles of 84 genes related to insulin-responsive genes were analysed using human insulin signalling pathway array. Of the 84 genes, 42 diabetic genes had at least a twofold change in expression for at least one comparison between the diabetic subjects, offspring with NGT and offspring with IGT as compared with controls. The most significant findings were that *FOXP3* and *SNAP25* were highly expressed in the offspring with IGT as compared with the controls, with a sixfold change in expression. The differential expression of the 42 genes among the offspring with IGT mainly demonstrates a defect in insulin secretion which suggests  $\beta$  cell dysfunction. The preponderance of experimental evidence favours the presence of

impaired rather than excessive insulin secretion in the offspring before the development of IGT and thus supports the concept that the initial lesion in type 2 diabetes may involve impaired insulin secretion rather than insulin resistance. The results from our study suggest that  $\beta$  cell dysfunction starts early in the pathologic process and does not necessarily follow the stage of insulin resistance.

**Keywords** Type 2 diabetics · Impaired glucose tolerance · Normal glucose tolerance · PBMCs · *FOXP3* · *SNAP25*

## Introduction

Type 2 diabetes (T2D) is known to result from the interaction of genetic and environmental factors [1]. The importance of genetics in the pathogenesis of T2D is indicated by several lines of evidence from studies of both twins and offspring of people with T2D [2]. In addition, T2D segregates in families, with substantial differences in prevalence between ethnic groups and races [2, 3]. Therefore, a predisposition to develop this disease is strongly determined by polygenic components, that is, the simultaneous presence of several abnormal genes or polymorphisms.

Although T2D is not clinically apparent until adulthood, metabolic abnormalities may be present and detectable much earlier. This suggests that even in apparently unaffected offspring of type 2 diabetic parents, abnormalities in insulin secretion and insulin sensitivity, each of which is under genetic control, may be present. Offspring of people with T2D, from here on referred to as just offspring, have a genetic predisposition to develop T2D and, thus, provide a good model in the search for the primary causes of the disease. It is well accepted that frank clinical T2D is preceded by a long prediabetic stage [4]. Impaired glucose tolerance (IGT) is a widely accepted

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entity of the prediabetic stage and is associated with hypertension, obesity, insulin resistance, and dyslipoproteinemia, commonly known as metabolic syndrome X [5, 6]. The role of genes in the etiology of abnormal glucose tolerance has been reported by Poulsen et al. [7]. Therefore, we suggest that genetic predisposition is important in the development of abnormal glucose tolerance.

Genomic approaches to the determination of differential expression profiles, using serial analysis of gene expression and DNA microarrays, are now providing global views of the potential genes and pathways that are associated with diabetes (Tables 1 and 2). With these approaches, tissue-specific gene expression analysis in human pancreas, muscle and fat has demonstrated the differential regulation of genes in diabetes [8]. Systemically circulating peripheral blood mononuclear cells (PBMCs) are considered a unique tissue affected by the host condition and may reflect oxidative stress caused by high levels of glucose, insulin, free fatty acids and tissue-derived circulating bioactive mediators [9]. Using a microarray hybridization approach, Choong-Chin Liew et al. [10] have shown that human peripheral blood cells express 80 % genes encoded in the human genome. They demonstrated that the genes previously restricted to non-blood tissues were expressed in peripheral blood cells and that their levels are indicative of their microenvironment. They also detected insulin in circulating blood cells and showed that environmental conditions affected the transcriptional regulation of insulin in the peripheral blood. Therefore, peripheral blood is an ideal surrogate tissue as it is readily obtainable. It also provides a large biosensor pool in the form of gene transcripts which responds to changes in the macro- and microenvironments and detectable as alterations in the levels of these gene transcripts. Kuppan Gogulakrishnan [11] using lymphocytes as a cellular model demonstrated an increase in subclinical inflammation/oxidation in Asian Indians with not only T2D but also IGT. Therefore, analysis of the genes in the PBMCs from offspring of one T2D parent may reflect genetic changes during the prediabetic stage.

To verify the hypothesis that the gene expression of PBMCs changes in response to diabetic circumstances, we comprehensively compared gene expression profiles of PBMCs among offspring with impaired glucose tolerance (IGT) and offspring with normal glucose tolerance (NGT) with that of patients with T2D and normal controls by using reverse transcription quantitative real-time polymerase chain reaction (PCR) array technology. Although there is extensive literature relating peripheral blood cells to diabetic complications, the association of these complications with the genes in PBMCs of offspring is unknown. To our knowledge, this is the first study on gene expression profiles in PBMCs of offspring with IGT and offspring with NGT. However, Jane Palsgaard has shown the differential expression of genes in

skeletal muscle biopsies from people with T2D and relatives [12].

The aim of this study was to demonstrate the possibility that gene expression in the PBMCs of the offspring with IGT may reflect genetic changes during the prediabetic stage.

## Materials and methods

### Subjects

Patients with T2D were randomly recruited through the diabetic clinic at the University of Malaya Medical Centre, Kuala Lumpur, and they consented to participate in this study. Subjects with a family history of diabetes, hypertension, coronary artery disease, and a body mass index of  $\pm 30 \text{ kg m}^{-2}$  were excluded from the study.

Offspring who had at least one parent with T2D without cardiovascular risk factors were chosen. The subjects were classified as having IGT if their fasting glucose level was  $\geq 6 \text{ mmol/l}$  and if their 2-h plasma post-glucose load (75 g) value was between 7.8 and 11 mmol/l. The subjects were classified as having NGT if their fasting glucose level was  $< 6 \text{ mmol/l}$  and their 2-h plasma glucose load value was  $< 7.8 \text{ mmol/l}$  in accordance with the limits set by the World Health Organization [13]. The present study comprised of 11 type 2 diabetic subjects who were newly diagnosed according to the American Diabetes Association protocol (Bloomgarden 1997) and recruited in the outpatient of diabetic clinic, Faculty of Medicine, University of Malaya. None of the subjects received hypolipidemic drug therapy or had any renal, hepatic or thyroid disease that affected glucose and lipid metabolism. Informed consent was obtained from all subjects, and the study was approved by the ethics committee of the University of Malaya Medical Centre.

### Analysis of blood samples

Fasting blood samples were collected in bottles containing disodium ethylene diaminetetraacetic acid (EDTA), and the plasma was separated immediately by centrifugation at 3000 rpm for 15 min at 40 °C. Total cholesterol, triglyceride and high-density lipoprotein levels were determined by using individual biochemical kits supplied with the Dimension R Clinical Chemistry System (Dode Behring, France), and low-density lipoprotein levels were determined by using the Friedewald equation [14]. Plasma samples were assayed at the same time for plasma glucose and HbA1C levels.

### Isolation of PBMCs

Blood was collected in EDTA tubes. PBMCs were isolated using the Ficoll density gradient method within 4 h of blood

**Table 1** Fold change in the differential expression of 84 diabetic genes for T2D subjects and offspring with NGT and IGT

No.	Symbol	Diabetes/control	IGT/control	NGT/control	No.	Symbol	Diabetes/control	IGT/control	NGT/control	No.	Symbol	Diabetes/control	IGT/control	NGT/control
1	ABCC8	-1.01	1.40	-1.11	31	IDE	-1.01	1.40	-1.11	61	PRKAA1	1.49	1.39	1.27
2	ACE	1.19	2.95	1.84	32	IFNG	1.74	1.40	1.29	62	PRKAG2	-1.16	1.40	1.27
3	ACLY	1.73	1.40	1.27	33	IGFBP5	1.11	1.39	1.72	63	PRKCB1	1.13	1.40	-1.11
4	ADRB3	1.13	2.80	1.80	34	IKBKB	-1.01	-1.43	-1.57	64	PTPN1	-1.16	1.40	1.79
5	AGT	-1.01	1.40	-1.56	35	IL10	1.31	1.40	1.80	65	PYGL	2.63	1.41	-1.56
6	AKT2	-2.02	-1.44	-1.11	36	IL12B	-1.32	1.42	-1.10	66	RAB4A	1.51	1.40	1.28
7	AQP2	1.30	2.80	1.80	37	IL4R	1.13	1.39	-1.11	67	RETN	1.99	2.79	1.81
8	CCL5	1.72	-1.44	1.80	38	IL6	1.50	2.81	1.27	68	SELL	2.28	1.40	-1.11
9	CCR2	1.98	1.40	1.27	39	INPPL1	1.31	2.80	1.28	69	SLC2A4	-1.01	2.76	1.80
10	CD28	1.31	2.79	1.28	40	INS	1.30	2.76	1.80	70	SNAP23	1.98	1.40	-1.11
11	CEACAM1	-1.33	-1.43	-1.56	41	INSR	1.50	2.78	1.80	71	SNAP25	1.98	5.55	1.80
12	CEBPA	1.31	1.39	-1.11	42	PDX1	1.51	2.82	1.28	72	SREBF1	1.50	2.80	1.81
13	CTLA4	1.51	2.81	1.81	43	IRS1	-1.01	1.40	1.26	73	STX4	-1.01	1.40	1.81
14	DUSP4	1.32	1.41	1.29	44	IRS2	-1.16	1.40	1.28	74	STXBP1	1.73	2.80	1.80
15	ENPP1	1.15	2.81	1.28	45	MAPK14	1.54	1.43	-1.09	75	STXBP2	1.50	1.41	1.82
16	FBP1	-2.65	-1.42	-1.56	46	MAPK8	-2.02	1.40	-1.11	76	HNF1B	1.14	2.81	-1.10
17	FOXC2	-1.00	2.80	-1.10	47	ME1	1.15	2.80	-1.10	77	TGFB1	1.14	2.81	2.57
18	FOXG1	1.31	2.79	1.81	48	NEUROD1	1.17	1.48	1.81	78	NKX2-1	1.31	2.80	1.27
19	FOXP3	2.28	5.63	2.57	49	NFKB1	1.50	1.39	1.27	79	TNF	-1.16	-1.43	-1.11
20	G6PC	-1.15	1.40	-1.61	50	NOS3	-1.15	1.40	-1.10	80	TNFRSF1A	1.14	1.39	-1.10
21	G6PD	3.47	2.81	2.56	51	NRF1	1.73	2.82	2.54	81	TRIB3	1.31	2.81	1.28
22	GCG	1.72	2.80	1.27	52	NSF	-1.15	-1.44	-1.10	82	VAMP3	1.52	1.41	-1.10
23	GCGR	1.17	1.47	1.85	53	PARP1	2.63	1.40	1.81	83	VAPA	1.31	1.39	1.80
24	GCK	1.14	-1.43	1.81	54	PIK3C2B	1.98	2.77	1.27	84	VEGFA	-1.33	2.79	1.28
25	GLPR	1.51	2.81	1.81	55	PIK3CD	1.73	2.81	1.28	85	B2M	1.14	1.39	-1.11
26	GPD1	-1.15	2.78	-1.61	56	PIK3R1	-1.54	1.40	1.80	86	HPRT1	1.49	1.39	1.27
27	GSK3B	-1.15	-1.41	-1.55	57	PPARA	1.74	2.83	1.82	87	RPL13A	-1.16	-1.43	-1.10
28	HMOX1	-1.16	-1.42	-2.21	58	PPARG	1.73	2.81	3.58	88	GAPDH	-1.16	-1.43	-1.11
29	HNF4A	1.12	2.80	1.80	59	PPARGC1A	1.72	2.80	1.83	89	ACTB	-1.27	1.05	-1.03
30	ICAM1	-1.52	2.81	2.56	60	PPARGC1B	-1.00	1.40	1.28					

IGT impaired glucose tolerance, NGT normal glucose tolerance

**Table 2** Standard deviation in fold change in the differential expression of 84 diabetic genes for T2D subjects and offspring with NGT and IGT

No	Symbol	Diabetes/control	IGT/control	NGT/control	No	Symbol	Diabetes/control	IGT/control	NGT/control	No	Symbol	Diabetes/control	IGT/control	NGT/control
1	ABCC8	0.46	0.25	0.22	31	IDE	0.46	0.57	0.22	61	PRKAA1	1.09	0.35	0.31
2	ACE	0.50	0.44	0.51	32	IFNG	0.37	0.43	0.32	62	PRKAG2	0.49	0.32	0.32
3	ACLY	0.99	0.39	0.97	33	IGFBP5	0.45	0.21	0.42	63	PRKCB1	0.57	0.12	0.22
4	ADRB3	0.43	0.34	0.43	34	IKBKB	0.46	0.28	0.16	64	PTPN1	0.49	0.29	0.43
5	AGT	0.46	0.17	0.16	35	IL10	0.38	0.31	0.45	65	PYGL	0.78	0.13	0.49
6	AKT2	0.23	0.08	0.69	36	IL12B	0.29	0.35	0.22	66	RAB4A	0.24	0.62	0.32
7	AQP2	0.39	0.76	0.44	37	IL4R	0.52	0.28	0.22	67	RETN	1.01	0.90	0.45
8	CCL5	0.80	0.39	0.43	38	IL6	0.57	0.66	0.31	68	SELL	1.04	0.22	0.22
9	CCR2	0.84	0.22	0.33	39	INPP1L	0.44	0.34	0.99	69	SLC2A4	0.36	0.47	0.44
10	CD28	0.39	0.51	0.32	40	INS	0.38	0.45	0.44	70	SNAF23	1.00	0.21	0.22
11	CEACAM1	0.55	0.39	0.49	41	INSR	0.57	0.73	0.43	71	SNAP25	0.91	0.82	0.44
12	CEBPA	0.39	0.18	0.22	42	PDX1	0.58	1.03	0.99	72	SREBF1	1.10	1.39	2.50
13	CTLA4	0.23	0.55	0.44	43	IRS1	0.46	0.52	0.95	73	STX4	0.46	0.36	0.44
14	DUSP4	0.39	0.17	0.99	44	IRS2	0.49	0.11	2.77	74	STXBP1	0.37	0.25	0.45
15	ENPP1	0.42	0.14	0.32	45	MAPK14	0.77	0.24	0.23	75	STXBP2	0.24	0.20	0.45
16	FBP1	0.14	0.17	0.16	46	MAPK8	0.23	0.94	0.22	76	HNF1B	0.42	1.14	0.22
17	FOXC2	0.46	0.59	0.22	47	ME1	0.76	0.92	0.22	77	TGFB1	1.25	0.76	1.98
18	FOXG1	0.67	0.13	0.45	48	NEUROD1	0.48	0.32	0.44	78	NKX2-1	0.45	0.57	0.33
19	FOXP3	0.85	0.77	1.96	49	NFKB1	0.24	0.18	0.32	79	TNF	0.19	0.38	0.22
20	G6PC	0.19	0.13	0.18	50	NOS3	0.54	0.20	0.22	80	TNFRSF1A	0.52	0.31	0.22
21	G6PD	1.98	0.21	1.97	51	NRF1	0.99	0.27	1.95	81	TRIB3	0.39	0.38	0.32
22	GCG	0.37	0.36	0.31	52	NSF	0.50	0.25	0.22	82	VAMP3	0.24	0.41	0.22
23	GCGR	0.47	0.70	0.53	53	PARP1	2.43	0.28	0.45	83	VAPA	0.39	0.15	0.45
24	GCK	0.49	0.16	0.44	54	PIK3C2B	0.92	0.86	0.97	84	VEGFA	0.60	0.31	0.98
25	GLP1R	0.58	0.18	0.44	55	PIK3CD	1.00	0.46	0.98	85	B2M	0.42	0.55	0.22
26	GPD1	0.19	0.34	0.18	56	PIK3R1	0.60	0.41	0.45	86	HPRT1	0.24	0.54	0.31
27	GSK3B	0.18	0.18	0.16	57	PPARA	0.99	0.37	0.45	87	RPL13A	0.49	0.64	0.22
28	HMOX1	0.40	0.15	0.98	58	PPARG	1.08	0.56	0.89	88	GAPDH	0.19	0.33	0.69
29	HNF4A	0.44	0.62	0.45	59	PPARGC1A	0.38	0.21	0.45	89	ACTB	0.43	0.12	0.23
30	ICAM1	0.19	0.84	0.62	60	PPARGC1B	0.46	0.26	0.97					

collection. If the cells were not processed immediately, they were lysed in buffer containing  $\beta$ -mercaptoethanol and stored at  $-80^{\circ}\text{C}$  for later processing.

### Isolation of RNA from PBMCs

Mononuclear cells were isolated by the Ficoll density gradient method, as described previously (Tateno et al., 2007). Total RNA was extracted with an RNA isolation kit (Qiagen, Rneasy MiniKit, USA), using the protocol described by the manufacturer. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

### PCR arrays

Double-stranded cDNA was generated from 0.5 to 1.0  $\mu\text{g}$  of total RNA, using a cDNA synthesis kit (SA Bioscience) that eliminated any contamination from DNA. cDNA was used as a template for a SYBR-green-based RT<sup>2</sup>-qPCR array using the diabetes array plate (SA Biosciences). Five housekeeping genes (*B2M*, *HPRT1*, *RPL 13A*, *GAPDH*, *ACTB*) and reverse transcription controls were used to normalize the gene expression studies.

The number of gene expression profiles consisted of five diabetic subjects versus one normal control, two offspring with normal glucose tolerance versus one normal control and two offspring with impaired glucose tolerance versus one normal control.

### Measurement of fold changes

Gene expression analysis was performed by using the human diabetes RT<sup>2</sup> Profiler<sup>TM</sup>. The expression of >30 and the blank were coded as 30. For each gene, the average of the expression levels, Ct, was computed. The difference in the average expression between genes of interest (GOI) and the housekeeping gene (HKG) was computed ( $Ct = Ct_{GOI} - Ct_{HKG}$ ). The normalized gene expression was computed as  $2^{-Ct}$ . To obtain the fold changes, we divided the normalized gene expression for the test group by that of the control group. A value of more than 1 indicates upregulation. A value of less than 1 indicates downregulation. For fold regulation comparison, the negative inverse of the fold change is considered from downregulated genes. For upregulated genes, the fold change was taken as the fold regulation value.

## Results and discussion

It has been reported that genes play a role in the etiology of abnormal glucose tolerance and that genetic predisposition is an important factor in the development of abnormal glucose tolerance. Non-genetic factors might, however, play a

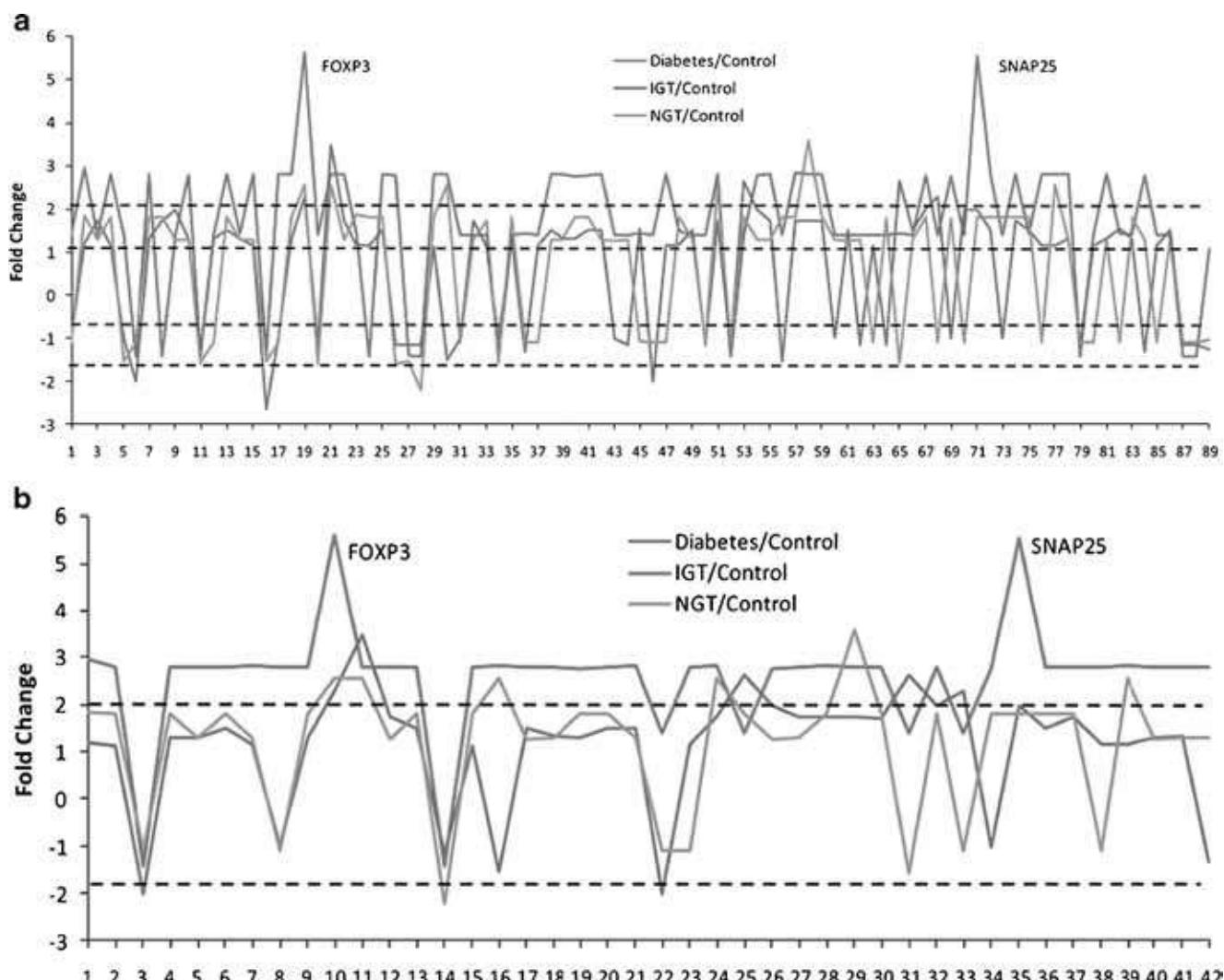
predominant role in controlling whether a genetically predisposed individual progresses to overt T2D [7]. Our work demonstrated a significant increase in total cholesterol and triglyceride levels in the offspring with IGT as compared to the offspring with NGT and with controls. Because of the scarcity of data in Malaysia on the pathogenesis of offspring from one type 2 diabetic parent and because genetics play an important role in T2D, we decided to study the pattern of genes in the offspring of one type 2 diabetic parent classified as having IGT versus healthy controls, in the offspring classified as having NGT versus healthy controls and in patients with T2D versus healthy controls. Until recently, gene expression in offspring of one type 2 diabetic parent with IGT has not been reported, which would enable us to see whether the genes expressed in the offspring with IGT is similar to that of subjects with T2D and to determine whether these genes may be involved in the progression of IGT to T2D.

In the following discussion, we include some of the significant genes with a twofold change in expression that are mainly involved in insulin resistance,  $\beta$  cell function and metabolic syndrome (Fig. 1).

The heat map in Fig. 2 of the offspring with IGT versus controls shows that fork head box P3 (*FOXP3*) and synaptosomal-associated protein of 25 kDa (*SNAP25*) were highly expressed in the offspring with IGT as compared to controls, with a sixfold change in the expression of *SNAP25* and *FOXP3*. Increased expression of *SNAP25* has been shown to induce impairment of insulin secretion in mouse islets [15]. Recent studies indicate that the overexpression of a mutant form of *SNAP25*, lacking the last nine residues, leads to ablation of cAMP-induced enhancement of  $\beta$  cell exocytosis [16]. Therefore, the overexpression of *SNAP25* as shown in our study could possibly reflect a defect in  $\beta$  cell exocytosis.

T regulatory cells (Tregs) are a component of the immune system that suppresses the immune responses of other cells. Tregs come in many forms, with the most well understood being those that express CD4, CD25 and *FOXP3* (CD4+ CD25+ Tregs). CD4+CD25+ T cells expressing forkhead transcription factor *Foxp3* are recognized as professional Tregs and are instrumental in the induction and maintenance of immune tolerance. Tregs make up 5–20 % of the CD4+ T cell compartment and are found within the major histocompatibility complex class II restricted CD4-expressing (CD4+) helper T cell population. They express high levels of interleukin 2 (IL-2) receptor  $\alpha$  chain CD25.

*FOXP3*+ Tregs differentiate during T cell development in the thymus, where the presentation of antigen by cortical or medullary thymic epithelial cells is sufficient to induce *FOXP3* expression in developing thymocytes [17]. Expression of *FOXP3* is required for Treg cell development and appears to control a genetic programme specifying this cell fate. *FOXP3* is not only a specific cell marker of Tregs but also a specific functional protein, the expression of which is



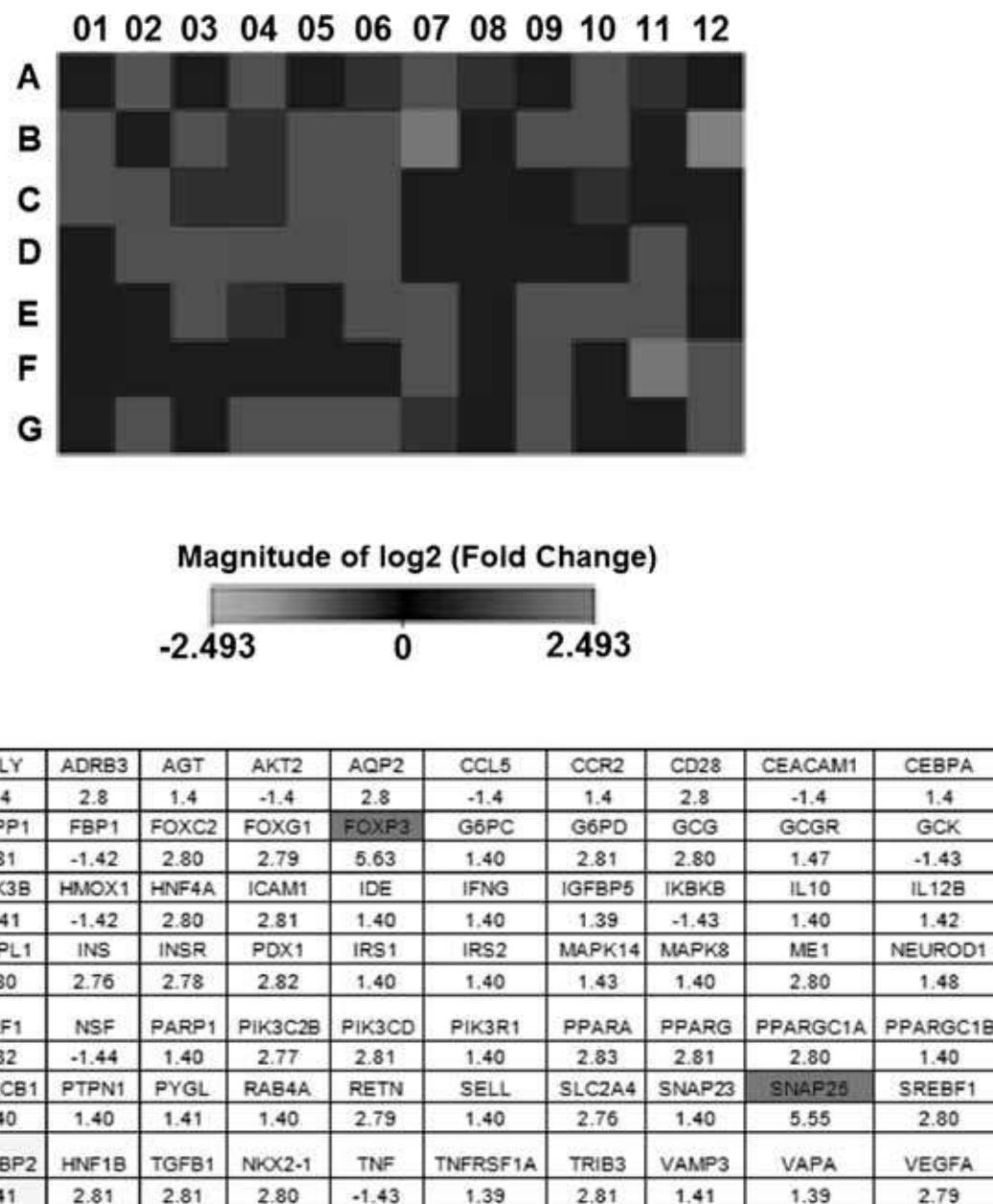
**Fig. 1** **a** Schematic representation comparing the fold changes in the expression of 84 diabetic genes for the diabetic, offspring with NGT and offspring with IGT subjects compared to the healthy controls. Based on this figure, the differential expressions for 42 genes were seen to have at least a twofold change for at least one of the comparisons. Differences were compared in diabetic subjects and first-degree relatives with normal glucose tolerance (NGT) and impaired glucose tolerance (IGT) as compared with controls. The networks of genes in **b** were

classified into different functional categories: nuclear receptors, signal transducers,  $\beta$  cell function and insulin secretion, adhesion molecules, metabolic enzymes, inflammatory genes, regulatory genes and transcription factors in gluconeogenesis and summarized in Table 3. Among the 89 genes, these had a fold change of at least two for at least one comparison between the diabetic subjects and the first-degree relatives with normal glucose tolerance (NGT) and impaired glucose tolerance (IGT) as compared with controls

the switch in Treg cell function (Table 3). The thymically derived Tregs retain a stable phenotype even after they are exported to the periphery. There they can become activated by a specific antigen and acquire some of the phenotypical characteristics of effector memory T cells. Therefore, this T cell subset has the defining features of a stable T cell lineage of immune response. CD4+CD25+*FOXP3* Tregs have been referred to as naturally occurring Tregs to distinguish them from the suppressor T cell population that is generated in vitro. In addition to their development intrathymically, CD4+CD25+*FOXP3* Tregs may also be converted extrathymically from CD4+CD25-naïve T cells through the induction of *FOXP3* by transforming growth factor beta (TGF $\beta$ ) [18, 19] (Table 4).

TGF- $\beta$ -dependent induction of *FOXP3* expression upon activation of peripheral T cells revealed important differences in epigenetic marks of the *FOXP3* locus in these cells compared with ex vivo isolated *FOXP3* Tregs. In the latter cells, an intronic CpG island within the conserved non-coding sequencing element is demethylated, consistent with the active *FOXP3* locus, whereas it remains largely methylated in TGF- $\beta$ -induced *FOXP3*+ T cells and in Foxp3-T cells and hence seemingly inactive [20, 21].

Recent data suggest that all CD4+memory T cells contain cells that have the capacity to produce IL-17 and that CD4+CD25<sup>high</sup> *FOXP3*+IL-17+T cells are Treg cells [22].



**Fig. 2** Differential expression of genes in first-degree relatives with impaired glucose tolerance versus controls

IL-17-producing CD4+ T-helper (Th17) cells have recently been defined as a unique subset of proinflammatory helper cells whose development depends on signalling initiated by IL-6 and TGF- $\beta$ , while IL-23 stabilises the generation of the Th17 subset. A study revealed that T2D-linked cytokine IL-6 increased the number of IL-17 cells in the spleen of obese mice, supporting the possibility that Th17 cells contribute to T2D-associated inflammation and insulin resistance [23]. Importantly, it has been demonstrated that T cell IL-17 production correlates with T2D severity, as measured by HbA1C, further highlighting a likely relationship between the Th17 T cell population and metabolic imbalance. Human thymus does not contain IL-17-producing Tregs, and this suggests that IL-

17+FOXP3 Tregs are generated in the periphery and that CD4+CD25<sup>high</sup>-derived T cell clones express FOXP3, ROR $\gamma$ t and IL-17. IL-17 upregulates specific chemokines, proinflammatory cytokines and colony-stimulating factors and is implicated in the pathogenesis of inflammation [24].

It has also been shown that the percentage of CD4+CD25+FOXP3 cells is significantly reduced in patients with newly diagnosed T2D compared with controls. Therefore, the patient with newly diagnosed T2D may have autoimmunoregulatory dysfunction, with elevated proinflammatory T cell subsets which are reinforced by the natural depletion of CD4+FOXP3 Tregs in the blood of T2D patients [23].

**Table 3** Fold change of at least two for at least one comparison (42 genes)

No	Symbol	Diabetes/ control	IGT/ control	NGT/ control	No	Symbol	Diabetes/ control	IGT/ control	NGT/ control
1	ACE	1.19	2.95	1.84	22	MAPK8	-2.02	1.40	-1.11
2	ADRB3	1.13	2.80	1.80	23	ME1	1.15	2.80	-1.10
3	AKT2	-2.02	-1.44	-1.11	24	NRF1	1.73	2.82	2.54
4	AQP2	1.30	2.80	1.80	25	PARP1	2.63	1.40	1.81
5	CD28	1.31	2.79	1.28	26	PIK3C2B	1.98	2.77	1.27
6	CTLA4	1.51	2.81	1.81	27	PIK3CD	1.73	2.81	1.28
7	ENPP1	1.15	2.81	1.28	28	PPARA	1.74	2.83	1.82
8	FOXC2	-1.00	2.80	-1.10	29	PPARG	1.73	2.81	3.58
9	FOXG1	1.31	2.79	1.81	30	PPARGC1A	1.72	2.80	1.83
10	FOXP3	2.28	5.63	2.57	31	PYGL	2.63	1.41	-1.56
11	G6PD	3.47	2.81	2.56	32	RETN	1.99	2.79	1.81
12	GCG	1.72	2.80	1.27	33	SELL	2.28	1.40	-1.11
13	GLP1R	1.51	2.81	1.81	34	SLC2A4	-1.01	2.76	1.80
14	HMOX1	-1.16	-1.42	-2.21	35	SNAP25	1.98	5.55	1.80
15	HNF4A	1.12	2.80	1.80	36	SREBF1	1.50	2.80	1.81
16	ICAM1	-1.52	2.81	2.56	37	STXBP1	1.73	2.80	1.80
17	IL6	1.50	2.81	1.27	38	HNF1B	1.14	2.81	-1.10
18	INPPL1	1.31	2.80	1.28	39	TGFB1	1.14	2.81	2.57
19	INS	1.30	2.76	1.80	40	NKX2-1	1.31	2.80	1.27
20	INSR	1.50	2.78	1.80	41	TRIB3	1.31	2.81	1.28
21	PDX1	1.51	2.82	1.28	42	VEGFA	-1.33	2.79	1.28

IGT impaired glucose tolerance, NGT normal glucose tolerance

Immune cell-mediated inflammation has been implicated in both the etiology and ongoing pathogenesis of insulin resistance and T2D. The ultimate goal of immunotherapy is therefore to achieve balance between effector T cells and Tregs, which not only benefits blood sugar control but also reduces the incidence of infection. The development of anti-lymphocyte therapies with selective action on pathogenic subsets or with the ability to promote protective Treg cell function will increase the available options for T2D treatment.

The fact that TGF $\beta$  and IL-6 are upregulated in the offspring with IGT may indicate that the *FOXP3* Treg cells that

were expressed could be inflammatory Th17 cells. This study demonstrates the expression of *FOXP3* in subjects with IGT, whose type and function can only be determined by analysing the *FOXP3* genes.

#### Nuclear receptors that were upregulated in offspring with IGT

The nuclear receptors that were upregulated in offspring with IGT are peroxisome proliferator-activated receptor gamma (*PPARG*) (2.81) and peroxisome proliferator-activated

**Table 4** Genes classified into broad functional categories

Functions	Genes
Nuclear receptor	PPARG, PPARGC1
Signal transducers	INSR, Akt2, SLC2A4, ENPP1, PIK3CD, MAPK pathway, INPPL1
Islet hormones and receptors	INS, GCG, GLP1R, GLP-1
B cell function and insulin secretion	Stxbp1, PDX1, Adrb3, Akt2, Aqp2, Cd28, Ctl4, GLP2R, SLC2A4, GCG, INSR, snap25, cbvPPARG, PPARGC1, PYGL, RETN, INS, MEL, NRF1, TG $\beta$ P1, HMOX1, Foxc2
Adhesion molecules	SELL, ICAM-1
Metabolic genes	PYGL, ENPP1, ACE, G6PD, MEL, PARP1
Inflammatory genes	IL-6, IL1 $\beta$ , CTLA4, PARP-1, TGF $\beta$ -1, HMOX-1, C28
Regulatory genes and transcription factors in gluconeogenesis	TRIB3, SLC2A4, HNF4A, snap25, GLUT4, PPARG, FOXP3, HMOX1, G6PD, AKT2, MAPK8, IRS1, IRS2, PRKM81P, SREBP-1, NKX21, HNFB1, FOXG1, PARP-1, INPPL1

receptor gamma co-activator 1 (*PPARGC1*) (2.80). PPAR $\gamma$  is a nuclear receptor that is indispensable in adipocyte differentiation and glucose homeostasis, and activated PPAR $\gamma$  in adipocytes is essential for the maintenance of whole body sensitivity [25, 26].

*PPARGC1A* is a nuclear hormone receptor co-activator that induces and coordinates the expression of genes involved in hepatic gluconeogenesis [26]. It also regulates adipocyte differentiation and glucose homeostasis and controls the peroxisomal beta-oxidation pathway of free fatty acids [27].

*PPARG* is expressed in pancreatic islets and is a downstream target of sterol regulatory element-binding protein 1C (*SREBP-1C*). It was upregulated only in the offspring with IGT (2.8). Both forms of *PPARG* are expressed in islets and are upregulated by the overexpression of the lipogenic transcription factor *srebp1c*. The principal response of *PPARG* is the activation of genes involved in the disposal, rather than the synthesis, of fatty acids. Although fatty acid oxidation may have beneficial effects on  $\beta$  cell function in the longer term by countering  $\beta$  cell “lipotoxicity”, the acute response to this metabolic shift is a marked inhibition of insulin secretion [28].

#### Expression of genes involved in the regulation of the insulin signalling pathway

Several noteworthy genes of the insulin signalling pathway, such as *INSR*, *SLC2A4* (glucose transporter4 [Glut 4]), *PIK3CD* (phosphoinositide 3 kinase catalytic delta polypeptide), *SNAP 25*, *STXBP1*, *ENPP1* (ectonucleotidylpyrophosphatase/phosphodiesterase 1), and *INPPL1*, had a twofold higher expression in the offspring with IGT than in the controls. There are however two potential insulin receptor-dependent signal transduction pathways. Insulin activates the PI3 kinase-dependent pathway that is necessary but not sufficient to induce GLUT 4 translocation. In parallel, the insulin receptor activates an additional pathway, leading to Cb1 tyrosine phosphorylation through its interaction with CAP protein, syntaxin 4 and PI3 kinase [29]. Similar to the results shown in our study for offspring with IGT, the findings by Lorella et al. [30] also showed that there was an increased expression of the insulin receptor *INSR* and *INPPL1* (inositol polyphosphate-5 phosphatase-like1). In the  $\beta$  cell tissues of subjects with T2D, however, Forkhead box 01 (*FOXO1*) was downregulated, while AKT-1, -2 and -3 all had strong expression with no differential expression.

#### Islet hormones and receptors

In our study, the array signals for insulin (*INS*), glucagon (*GCG*) and glucagon-like peptide 1 receptor (*GLP1R*) were twofold higher in the offspring with IGT. GLP1R receptors

are present in pancreatic periductal cells and  $\beta$  cells. GLP1R knock-out mice have abnormal glucose tolerance and fasting hyperglycaemia but are not obese, and GLP-1 [31] releases glucose-dependent insulin from the pancreatic islets.

#### Genes involved in $\beta$ cell function and insulin secretion

The expression of [*Stxbp1*] syntaxin binding protein 1 was upregulated more than twofold in the offspring with IGT. This gene plays an important role in the fusion of insulin secretory granules in the plasma membrane of the  $\beta$  cells of the islets and plays a part in the secretion of insulin. *PDX1*, pancreatic duodenal homeobox, is one of the transcription factors that activate insulin gene expression in response to increasing glucose levels. The production and secretion of insulin from  $\beta$  cells of the pancreas is crucial in maintaining normoglycaemia.

An increased expression of insulin signalling molecules in offspring with IGT in concert with increased levels of insulin as a compensatory mechanism counteracts otherwise reduced insulin signalling activity, protecting these individuals from severe insulin resistance. The compensation is lost in people with T2D, where expression of insulin signalling molecules is reduced.

#### Adhesion molecules that were upregulated in the offspring with IGT

The expression of intracellular adhesion molecule 1 (*ICAM-1*) (2.81) is increased in the offspring with IGT. Adhesion molecules recruit circulating leukocytes to the site of atherosclerosis, and their levels in the circulation may serve as molecular markers of subclinical coronary artery disease in T2D [32].

#### Metabolic genes involved in glucose homeostasis and lipid metabolism

The major gene that was upregulated in the offspring with IGT was glucose 6 phosphate dehydrogenase (*G6PD*). Its overexpression in adipocytes brings about oxidative stress and inflammation and is associated with insulin resistance, and this regulates the level of blood glucose [33]. Malic enzyme was increased in the offspring with IGT (2.80). The pyruvate malate shuttle is a metabolic cycle in pancreatic  $\beta$  cells and is important for  $\beta$  cell function. In rat islets, the pyruvate malate shuttle may regulate insulin secretion and has been shown to play a critical role in adaptation to obesity and insulin resistance [34].

The expression of human *INS*, which encodes the precursor to the hormone insulin, was increased twofold. Expression of the liver glycogen phosphorylase gene, *PYGL*, which breaks glycogen into glucose subunits, was increased twofold.

## Inflammatory genes

*IL-6* was increased in the offspring with NGT. It has been shown that glucose-induced *IL-1 $\beta$*  upregulated the inflammatory factors IL-8 and IL-6 [12, 35]. The *CTLA4* gene mediates antigen-specific apoptosis and progressive  $\beta$  cell failure, which is a typical feature of T2D [36]. Recent data suggested that the apoptosis mechanism might explain insulin deficiency through a reduction in the absolute number of pancreatic  $\beta$  cells [37, 38].

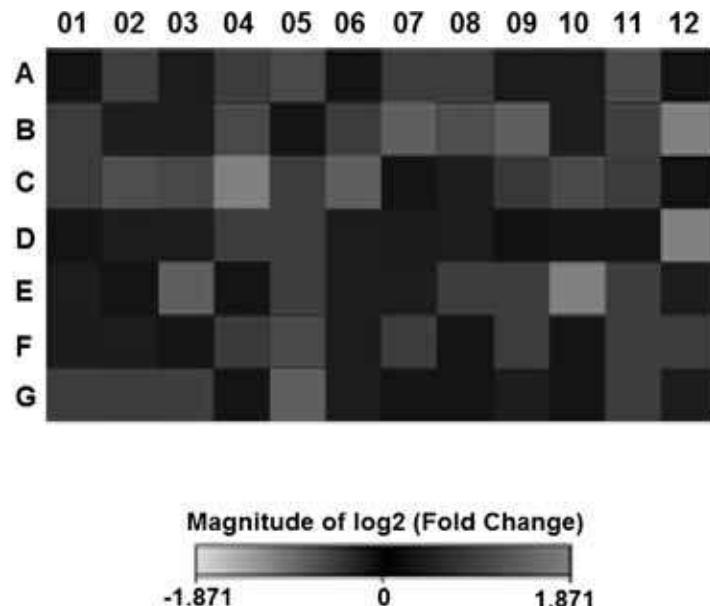
## Hepatic gluconeogenesis

Expression of representative genes for hepatic gluconeogenesis such as *PPARGC1A*, Tribbles homolog 3 (*TRIB3*), solute carrier family 2 (*SLC2A4*) and *HNF4A* was twofold higher in

the offspring with IGT as compared with controls. Upregulation of these genes may provide a molecular basis for elevated gluconeogenesis in the livers of offspring with IGT. T2D is characterized by impairment in the ability of insulin to inhibit gluconeogenesis in the liver [39].

*SNAP25* and *Syntaxin1*, which are upregulated in offspring with IGT, form a complex that resides on the *GLUT4* vesicle surface and plays an important role in the interaction between the vesicle and the plasma membrane surface. An increase in the expression of proteins promoting efficient *GLUT4* trafficking and fusion to the membrane, such as *SNAP25*, could compensate for a decreased amount of *GLUT4* in diabetic patients.

The heat map and table of genes show that the expression of some important genes is increased more than twofold in the offspring with NGT (Fig. 3). PPARG had an increase of 3.7-



**Fig. 3** Differential expression of genes in first-degree relatives with normal glucose tolerance versus controls

fold as compared with controls. The other two genes that were increased were *FOXP3* (2.62-fold) and *TGFβ* (2.62-fold). *TGFβ* is possibly involved in the regulation of *FOXP3*. The expression of *ICAM-1*, the marker of early coronary disease, was increased 2.62-fold.

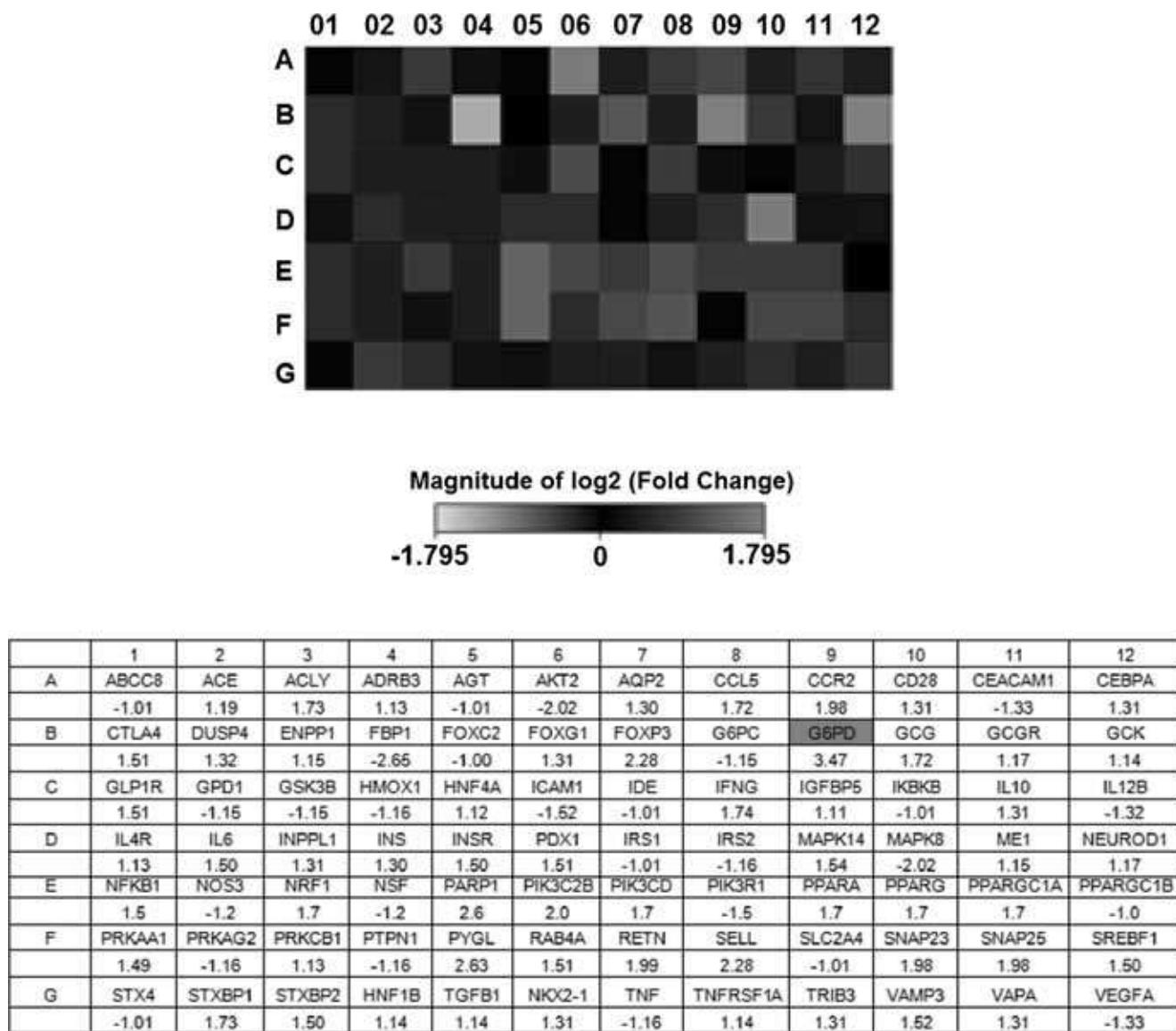
The key metabolic enzyme that was upregulated in offspring with NGT was glucose 6 phosphate dehydrogenase. *NRF1* was increased 2.6-fold which, by interacting with nuclear factor *PPARG C1A*, regulates mitochondrial gene transcription through the upregulation of mitochondrial transcription factor [38]. The gene hemeoxygenase 1 (*HMOX1*) was downregulated by twofold in the offspring with NGT. *HMOX1* is important for protection against oxidative stress, which suggests that β cells in T2D are chronically subjected to oxidative stress.

The heat map and table of genes in the subjects recently diagnosed with T2D versus controls (Fig. 4) show that two

metabolic genes were upregulated in the diabetic subjects. Glucose 6 phosphate dehydrogenase *G6PD* (3.47) and liver glycogen phosphorylase gene *PYGL* (2.63). In addition, there was an increased expression of *FOXP3* (2.28).

The most important gene that was downregulated less than twofold in patients with T2D was *Akt2*. These gene codes for a protein kinase and is thought to be a key mediator in the insulin signal transduction process. In addition, *AKT2* is involved in glucose metabolism and maintenance of proper adipose tissue and islet mass.

In addition, *MAPK8* was downregulated twofold in patients with T2D. The downregulation of *MAPK8* (2.02) could lead to a decreased serine/threonine phosphorylation of insulin receptor substrate (IRS) proteins, ultimately increasing signalling activity as part of a compensatory mechanism directed against insulin resistance. *MAPK8* also regulates β cell



**Fig. 4** Differential expression of genes of patients with newly diagnosed type 2 diabetes versus controls

function and glucose transporter 2 and acts as an antiapoptotic protein of  $\beta$  cells [40].

Besides some important genes such as SLC2A4, IRS-1 and IRS-2 involved in insulin signalling pathway were downregulated in T2D patients, although not by twofold, whereas selectin and PARP-1 were upregulated. PYGL, the liver glycogen phosphorylase gene that breaks down glycogen to form glucose, was upregulated (2.63).

The interesting finding in our study is that the major metabolic genes glucose phosphate dehydrogenase and *FOXP3* were upregulated in the offspring with NGT. Both are involved in the inflammatory pathway, which suggests that the immune system plays an important role in the development of T2D. PPARG, an important gene, was upregulated in the offspring with NGT.

The underlying genetics of T2D is very complex, and it is clear that several genes play a role in making this a polygenic disease, including those shown in our work. Further, there are several different combinations of diabetogenes that can lead to T2D under the influence of certain environmental conditions [41].

Although there is a general agreement that subjects with type 2 diabetes have overt  $\beta$  cell dysfunction, the stage in the evolution of glucose intolerance at which this dysfunction develops is uncertain. The findings of our study suggest that the major factor responsible for the transition from NGT to IGT is the superimposition of insulin resistance upon impaired  $\beta$  cell function and that the major factor responsible for transition from IGT to T2D is the worsening of the already impaired insulin secretion.

Our study supports the data which had been reported previously by Bacha et al. where he demonstrated that  $\beta$  cell dysfunction develops early in the pathological process and does not necessarily follow the stage of insulin resistance in obese adolescents [42].  $\beta$  cell dysfunction as a major factor across the spectrum of prediabetes to diabetes is also stressed in adults. Cross sectional data from the study on risk factor in impaired glucose tolerance for atherosclerosis and diabetes demonstrated that isolated impaired fasting glucose and impaired glucose tolerance are different with respect to the degree of insulin resistance and anomalies in insulin secretion and that subjects with IGT exhibit a deficit in the early and late phases of insulin secretion. Another study demonstrated that early stages of glucose intolerance are associated with disturbances in  $\beta$  cell function, while insulin resistance is seen more markedly in later stages [43].

The transition from IGT to type 2 diabetes could represent progression of a genetic  $\beta$  cell deficit and the toxic effects of hyperglycaemia. In most individuals, multiple genetic defects in insulin secretion may be necessary, but not sufficient, to cause diabetes without acquired factors such as the superimposition of insulin resistance (weight gain, glucose toxicity,

physical inactivity) or without the simultaneous presence of diabetes-related genes or diabetogenic genes, causing insulin resistance or predisposing the individual to it. Also, further deterioration in insulin sensitivity or secretion may enhance the risk for this progression.

Our study demonstrates that gene expressions are different at different stages of development, from the offspring of one diabetic parent with NGT to IGT to diabetes, demonstrating progressive  $\beta$  cell dysfunction and insulin resistance.

## Conclusion

The preponderance of experimental evidence favours the presence of impaired rather than excessive insulin secretion in the offspring of one type 2 diabetic parent before the development of IGT and thus provides support for the concept that the initial lesion in T2D may involve impaired insulin secretion rather than insulin resistance. Therefore, singular focus on insulin resistance as the “be all and end all” is gradually shifting, and hopefully better treatment options that address  $\beta$  cell pathology will emerge for early therapy.

Our data suggest that measures to prevent progression or conversion from prediabetes to type 2 diabetes should target improvement on  $\beta$  cell function.

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# A randomized controlled clinical trial of combination therapy for type 2 diabetes by vildagliptin, metformin, and $\alpha$ -glucosidase inhibitor

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**Abstract** This study aims to evaluate the efficacy and safety of vildagliptin combined with metformin and  $\alpha$ -glucosidase inhibitors for treatment of type 2 diabetes. The type 2 diabetic patients with poor blood sugar control after a combination treatment by metformin and  $\alpha$ -glucosidase inhibitor for at least two months were randomly assigned to receive vildagliptin or placebo (100 mg/day for 2 times) on the basis of the original treatment. The relevant indicators of the patients before and after the test for 12 weeks were detected. We totally chose 490 cases of subjects who were in Henan Provincial People's Hospital from February, 2013 to July, 2013. Compared with the data before the test, the glycated hemoglobin (HbA1c) changed from  $8.86 \pm 1.820$  to  $6.74 \pm 1.256$  % and fasting blood glucose (FBG), postprandial blood glucose (PPG), and alanine aminotransferase (ALT) in the vildagliptin group significantly decreased; the difference was statistically significant ( $P < 0.05$ ). The differences of the index in the placebo group were not statistically significant ( $P > 0.05$ ). HbA1c in the vildagliptin group, 12 weeks after the test, was significantly lower than those in the placebo group with a comparison by  $6.74 \pm 1.256$  and  $8.20 \pm 1.180$  %, as well as FBG, PPG, ALT, and aspartate aminotransferase (AST); the difference was statistically significant ( $P < 0.05$ ). Whether the comparisons were performed before and after treatment or between the two groups, the changes of the subject's body weight and blood

lipids had no statistically significant differences ( $P > 0.05$ ). Vildagliptin combined with metformin and  $\alpha$ -glucosidase inhibitors can efficiently reduce the FBG, PPG, and HbA1c of the patients without gaining weight and may have a protective effect on the liver.

**Keywords** Type 2 diabetes · Vildagliptin · Metformin ·  $\alpha$ -Glucosidase inhibitors

## Introduction

Type 2 diabetes is characterized by insulin resistance with a performance of pancreatic  $\beta$ -cell progressive failure, leading to a lack of insulin secretion after glucose loading and blood glucose levels increasing. Long-term hyperglycemia will lead to serious complications, and effective glycemic control can significantly reduce the incidence of diabetic complications and mortality [1]. In recent years, dipeptidyl peptidase-IV (DPP-IV) inhibitors are becoming new research targets for treatment of type 2 diabetes.

Vildagliptin is a potent and selective inhibitor of DPP-IV. Studies have shown that vildagliptin can significantly low the blood glucose and glycated hemoglobin (HbA1c) levels in patients with type 2 diabetes, both alone or combined with oral hypoglycemic drugs or insulin are valid [2–8].

However, the combined use of vildagliptin and metformin glycosidase inhibitors is not reported yet. Therefore, the clinical trial was designed. The purpose of this study was to compare the efficacy of vildagliptin and placebo in type 2 diabetes patients with poor glycemic control after being treated by metformin and  $\alpha$ -glucosidase inhibitors, and whether adverse reactions after using vildagliptin was observed.

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## Subjects and methods

### Subjects

Inclusion criteria are as follows: (1) type 2 diabetic subjects determined by the results of 75 g oral glucose tolerance test (OGTT) (2010 ADA diagnostic criteria), (2) aged from 18 to 70 years old, (3) used metformin and  $\alpha$ -glucosidase inhibitors (acarbose) for over 2 months, (4) HbA1c > 6.5 %, fasting blood glucose (FBG) > 7.0 mmol/L, (5) kidney function was normal, and (6) voluntary to participate in this study and signed an informed consent form.

Exclusion criteria are as follows: (1) type 1 or other specific types of diabetes, (2) age < 18-year old or > 70-year old, (3) in addition to metformin and  $\alpha$ -glucosidase inhibitors, combined use of other hypoglycemic drugs, (4) diabetic ketoacidosis poisoning, nonketotic hyperosmolar syndrome, or chronic complications which required that insulin therapy must be used under stress, (5) overt hepatic and renal disease (alanine aminotransferase (ALT) or aspartate aminotransferase (AST) were greater than three times of the upper limit of normal, or TBIL greater than one and a half times of the upper limit of normal, or Cr > 115  $\mu$ mol/L), (6) pregnant or lactating women, and (7) bad compliance. This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Henan Provincial People's Hospital. Written informed consent was obtained from all participants.

### Test design

This was a trial without being registered because we just enrolled participants in one hospital, and the study period was relatively short. Drug providers provided vildagliptin and the placebo, randomized numbers to the drugs, and put the numbers on the drugs saving as blind codes. Doctors enrolled participants and assigned participants to interventions. Patients brought into the test got the drugs according to the order of joining in. Dosage was 100 mg in one day, taking in two times. After 12 weeks, they crosschecked all case reports and confirmed and locked the data. Then they exposed the blind for the first time, distinguished between the two groups. Statistical analysis was performed by professional statisticians, and they put forward the report. Once confirmed, they exposed the blind for the second time, distinguished between the vildagliptin and the placebo groups.

### Development of a diet program

Individualized dietary guidance was performed for all subjects by a specialized endocrinologist. During the experiment, according to the ideal body weight of the daily per kilogram (ideal body weight (kg)=measured weight (kg)–105), about

30 kcal was provided to each subject, including approximately 50 % carbohydrate, 15 % protein, 35 % fat and assigned to three meals per day according to 1/5, 2/5, and 2/5. The meal-time and content were required to be relatively uniform. Tea or coffee was forbidden during the experiment.

### Medication

According to the randomized placebo-controlled trial design, the selected subjects were randomly distributed with vildagliptin (100 mg/day for 2 times) and placebo. Subjects were required to take prescribed medication during the test requirements; subjects with missing taking, adjusting dose, or treatment programs were deemed to have withdrawn. Fingertip blood glucose and whether hypoglycemic events existed were self-monitored and recorded by the tested patients. The body weight, FBG, postprandial blood glucose (PPG), HbA1c, blood lipid, and liver and kidney functions were retested in the hospital 12 weeks later. All drugs used in the test were provided by the Henan Pharmaceutical Co., Ltd., Warehouse Street 149, 450000, Zhengzhou City, Henan Province.

### Relevant indicators

Relevant indicators include general information (such as age, gender, height, and weight); blood glucose (FBG, PPG) which was measured by a glucometer of Johnson & Johnson (JNJ) using glucose oxidase method (GOPOD); HbA1c, measured by Bio-Rad D-10-type analyzer using ion exchange chromatography high-pressure liquid chromatography; and biochemical indicators (ALT, AST, CHOL, TG, UREA, CREA), measured by Nissan 7600-type full automatic biochemical analyzer.

### Statistical analysis

Data were expressed as mean $\pm$ standard deviation. The comparison for gender composition of the two groups was completed by chi-square test. Blood glucose, biochemical indicators, and weight were firstly tested by the normality test and homoscedasticity test. If they conform to both of the two tests, comparisons of data before and after treatment in the two groups will be performed using *t* test. If the data is still not satisfied with normality and variance after transformation, then nonparametric statistics will be used. All statistics are conducted by SPSS13.0 software. A *P* value of <0.05 was considered to be statistically significant.

## Results

### Clinical features of subjects

A total of 490 cases of subjects (276 male and 214 female), including 245 cases for the vildagliptin group and 245 cases for the placebo group, aged  $46.14 \pm 14.03$  (18~70) years old, weighed  $66.90 \pm 10.100$  (45~80) kg, with FBG of  $9.13 \pm 1.621$  (7.0~12.0) mmol/L, mean postprandial glucose (MPPG) of  $10.49 \pm 1.577$  (7.0~13.0) mmol/L, and HbA1c of  $8.74 \pm 1.599$  (6.5~11.6) %. The flow diagram of this trial was shown in Fig. 1. And the difference between the two groups about related indicators such as age, body weight, FBG, MPPG, HbA1c, lipid, and liver and kidney functions of the patients was shown in Table 1.

### Comparisons of related indicators before and after treatment

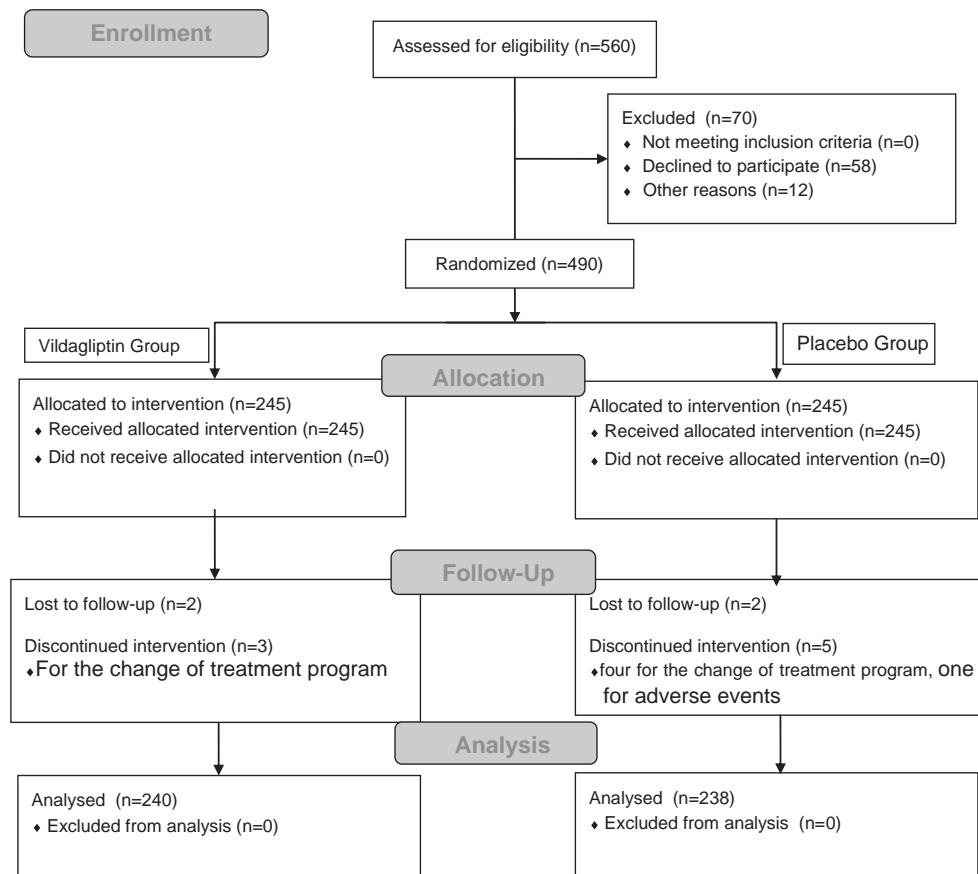
Twelve weeks after the test, the results in Table 2 showed that FBG, MPPG, HbA1c, and ALT in the vildagliptin group were significantly decreased compared with the previous, and the difference was

**Table 1** Clinical characteristics of the subjects ( $\bar{x} \pm s$ )

Item	Group vildagliptin (n=240, male to female ratio= 139:101)	Group placebo (n=238, male to female ratio=130:108)	P value
Age (years)	$46.61 \pm 15.41$	$45.67 \pm 12.65$	0.84
BMI (kg/m <sup>2</sup> )	$24.06 \pm 8.38$	$24.27 \pm 8.17$	0.54
Duration of diabetes (years)	$8.09 \pm 5.73$	$8.17 \pm 6.01$	0.60
FBG (mmol/L)	$9.36 \pm 1.70$	$8.90 \pm 1.55$	0.42
MPPG (mmol/L)	$10.40 \pm 1.66$	$10.59 \pm 1.50$	0.99
HbA1c (%)	$8.86 \pm 1.82$	$8.62 \pm 1.38$	0.69
ALT (U/L)	$30.44 \pm 15.85$	$23.28 \pm 9.93$	0.11
AST (U/L)	$24.70 \pm 7.68$	$23.68 \pm 5.24$	0.33
CHOL (mmol/L)	$4.98 \pm 1.02$	$4.73 \pm 1.29$	0.34
TG (mmol/L)	$1.97 \pm 1.39$	$1.94 \pm 1.34$	0.86
HDL (mmol/L)	$1.20 \pm 0.31$	$1.04 \pm 0.28$	0.17
LDL (mmol/L)	$2.89 \pm 0.66$	$2.60 \pm 0.83$	0.23
UREA (mmol/L)	$5.59 \pm 1.65$	$5.68 \pm 1.27$	0.86
CREA (μmol/L)	$59.28 \pm 13.37$	$60.76 \pm 20.36$	0.57

*FBG* fasting blood glucose, *MPPG* mean postprandial glucose, *HbA1c* glycated hemoglobin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *CHOL* total cholesterol, *TG* triglycerides, *HDL* high-density lipoproteins, *LDL* low-density lipoproteins, *UREA* urea, *CREA* creatinine

**Fig. 1** Flow diagram



**Table 2** Comparisons of related indicators before and after vildagliptin and placebo treatment ( $\bar{x} \pm s$ )

Group	Item	Before treatment ( $\bar{x} \pm s$ )	After treatment ( $\bar{x} \pm s$ )	P value
Vildagliptin (n=240)	Weight (kg)	66.14±10.31	65.86±10.55	0.96
	FBG (mmol/L)	9.36±1.07	6.89±0.70	<0.0001*
	MPPG (mmol/L)	10.40±1.66	8.23±0.83	<0.0001*
	HbA1c (%)	8.86±1.82	6.74±1.26	<0.0001*
	ALT (U/L)	30.44±15.85	20.88±8.13	0.03*
	AST (U/L)	24.70±7.68	20.34±4.89	0.32
	CHOL (mmol/L)	4.98±1.02	4.50±0.88	0.15
	TG (mmol/L)	1.97±1.39	1.40±0.84	0.13
	HDL (mmol/L)	1.20±0.31	1.15±0.24	0.71
	LDL (mmol/L)	2.89±0.66	2.58±0.75	0.21
	UREA (mmol/L)	5.59±1.65	5.90±2.09	0.72
	CREA (μmol/L)	59.28±13.37	57.11±14.60	0.87
Placebo (n=238)	Weight (kg)	67.67±9.89	65.54±9.36	1.00
	FBG (mmol/L)	8.90±1.55	8.60±1.17	0.57
	MPPG (mmol/L)	10.59±1.50	9.70±1.34	0.14
	HbA1c (%)	8.62±1.38	8.20±1.18	0.30
	ALT (U/L)	23.28±9.93	26.79±8.07	0.24
	AST (U/L)	23.68±5.24	23.87±4.46	0.08
	CHOL (mmol/L)	4.73±1.29	4.67±0.95	0.92
	TG (mmol/L)	1.94±1.34	1.98±2.33	0.93
	HDL (mmol/L)	1.04±0.28	1.23±0.30	0.06
	LDL (mmol/L)	2.60±0.83	2.70±0.81	0.68
	UREA (mmol/L)	5.68±1.27	5.24±1.52	0.37
	CREA (μmol/L)	60.76±20.36	63.98±21.85	0.60

FBG fasting blood glucose, MPPG mean postprandial glucose, HbA1c glycated hemoglobin, ALT alanine aminotransferase, AST aspartate aminotransferase, CHOL total cholesterol, TG triglycerides, HDL high-density lipoproteins, LDL low-density lipoproteins, UREA urea, CREA creatinine

\*P<0.05

statistically significant ( $P<0.05$ ); the weight, lipids, and serum creatinine levels of the subjects showed no significant difference comparing with the data before the experiment ( $P>0.05$ ). The indicators in the placebo group were not statistically different indicators comparing with the data before the experiment.

#### Comparisons of results in the vildagliptin and placebo treatment groups

In the 12-week test, FBG ( $P<0.0001$ ), MPPG ( $P<0.0001$ ), and HbA1c ( $P=0.001$ ) in the vildagliptin group were significantly reduced compared with that in the placebo group; ALT and AST also declined, and the differences were significant. The differences of weight, blood lipids, and serum creatinine levels between the subjects in the two groups were not statistically significant (Table 3).

#### Adverse events in the vildagliptin and placebo treatment groups

Twelve subjects withdrew from the study in half, of which three cases in the vildagliptin group withdrew due to the change of treatment program, and two cases were lost contact. Four patients in the placebo group quit due to the change of treatment program, two patients were lost contact, and one case for adverse events. Two hundred forty patients in the vildagliptin group and 238 cases in the placebo group finally completed the study. Finally, there were two cases of diarrhea, one case of bellyache, one case of hypoglycemia, and one case of weight gain in the vildagliptin group while there was one case of diarrhea, one case of bellyache, one case of constipation, one case of hypoglycemia, and two cases of weight gain in the placebo group (Table 4).

**Table 3** Several important indicators after being treated by vildagliptin and placebo for 12 weeks ( $\bar{x} \pm s$ )

Item	Group vildagliptin (n=240)	Group placebo (n=238)	P value	95 % confidence intervals
Weight (kg)	0.28±10.43	2.13±9.63	0.72	(−4.41,0.71)
FBG (mmol/L)	2.47±1.20	0.30±1.36	<0.0001*	(1.83,2.51)
MPPG (mmol/L)	2.17±1.24	0.89±1.42	<0.0001*	(0.94,1.62)
HbA1c (%)	2.12±1.54	0.42±1.28	0.001*	(1.35,2.05)
ALT (U/L)	9.56±11.97	−3.51±9.00	0.03*	(10.39,15.75)
AST (U/L)	4.36±6.28	−0.19±4.85	0.02*	(3.13,5.97)
CHOL (mmol/L)	0.48±0.95	0.06±1.12	0.58	(0.16,0.68)
TG (mmol/L)	0.57±1.12	−0.04±1.84	0.09	(0.24,0.98)
HDL (mmol/L)	0.05±0.28	−0.19±0.29	0.34	(0.16,0.32)
LDL (mmol/L)	0.31±0.71	−0.10±0.82	0.61	(0.21,0.61)
UREA (mmol/L)	−0.31±1.87	0.44±1.39	0.37	(−1.16,−0.34)
CREA (μmol/L)	2.17±13.99	−3.22±21.10	0.25	(0.91,9.87)

FBG fasting blood glucose, MPPG mean postprandial glucose, HbA1c glycated hemoglobin, ALT alanine aminotransferase, AST aspartate aminotransferase, CHOL total cholesterol, TG triglycerides, HDL high-density lipoproteins, LDL low-density lipoproteins, UREA urea, CREA creatinine

\*P<0.05

## Discussion

As new oral hypoglycemic drugs, vildagliptin was a highly selective inhibitor of DPP-IV, which was capable of specifically inhibiting DPP-IV, could increase glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic (GIP) levels, promote insulin secretion, inhibit glucagon release, delay gastric emptying, and improve B cell function to prevent apoptosis [9–12] with advantages such as glycemic control without increasing body weight and causing low blood sugar [13]. Hypoglycemia and suppression of glucagon secretion were correlated with glucose [14] with mild adverse reactions and

good safety [15, 16]. At present, metformin has been widely recommended as the cornerstone of therapy for type 2 diabetes;  $\alpha$ -glucosidase inhibitor drugs were proved to have good efficacy and safety in terms of reducing postprandial hyperglycemia and may contribute to the GLP-1 secretion [17, 18].

Multiple randomized controlled trials showed that vildagliptin can significantly reduce blood glucose and HbA1c levels in patients with type 2 diabetes; it was effective whether alone or in combination with oral hypoglycemic drugs or insulin [2–8]. But studies on vildagliptin combined with metformin and glucosidase inhibitors have not yet been reported; this clinical trial was designed on this basis. The purpose of this experiment was to compare the efficacy of vildagliptin and placebo in poor glycemic control of patients with type 2 diabetes by oral metformin and  $\alpha$ -glucosidase inhibitors, whether adverse reactions after using vildagliptin was observed. Five people withdrew from the study in the vildagliptin group, one person had symptoms of hypoglycemia, seven people withdrew from the study in the placebo group, and one person had the symptoms of hypoglycemia.

The test results in this study showed that vildagliptin can significantly reduce FBG, PPG, and HbA1c levels and lead to a decrease of ALT levels; the weight change of the patient before and after treatment was not statistically significant.

Vildagliptin can significantly reduce FBG, PPG, and HbA1c levels, suggesting that vildagliptin had good effect for fasting and postprandial blood glucose control. As reported in the literature, vildagliptin monotherapy could reduce HbA1c for 0.5 to 1.0 % [19]. These results in the paper were caused by the combined effects of the vildagliptin and metformin and  $\alpha$ -glucosidase inhibitors, so the data was slightly different.

**Table 4** Comparison of AEs in each group

AEs	Group vildagliptin (n=240)	Group placebo (n=238)
Diarrhea	2	1
Bellyache	1	1
Constipation	0	1
Nausea	0	0
Vomit	0	0
Weakness	0	0
Upper respiratory tract infection	0	0
Severe pain	0	0
Back pain	0	0
Hypertension	0	0
Hypoglycemia	1	1
Weight gain	1	2

AEs adverse events

According to reports, many of the existing treatment options for type 2 diabetes patients can lead to weight gain [20]. The results of this study showed that the change in patient's weight before and after vildagliptin treatment was not statistically significant, that is, the risk of weight gain by vildagliptin treatment was lower.

The results also showed that vildagliptin can lead to low levels of ALT. A meta-analysis result of a clinical study for 38 II/III staging showed that vildagliptin was irrelevant with the risk of liver incident and liver enzymes increase [21], but no relevant study about vildagliptin resulting in decrease of ALT was reported. The reduction of liver enzyme levels in this test results may be associated with a small number of samples or the combined effects of oral hypoglycemic drugs.

However, the sample size of this trial was small and the scope was limited. It will be better to enlarge the sample size and increase the sample selection area. Another limitation of this trial was that its period was short; therefore, we could not evaluate the long-term curative effect of this combination.

In summary, vildagliptin in combination with metformin and  $\alpha$ -glucosidase inhibitors can reduce FBG, PPG, and HbA1c of type 2 diabetes without the risk of gaining weight and may have a protective effect on the liver. From the viewpoint of both safety and efficacy, vildagliptin combined with metformin and  $\alpha$ -glucosidase inhibitor was an ideal glucose control program for treatment of type 2 diabetics.

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ORIGINAL ARTICLE

# Co-infusion of insulin-secreting adipose tissue-derived mesenchymal stem cells and hematopoietic stem cells: novel approach to management of type 1 diabetes mellitus

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**Abstract** Stem cell therapy (SCT) has promising results in regeneration of injured tissues/cells as well as correcting immune dysregulation. We present our experience of co-infusion of human adipose tissue-derived insulin-secreting mesenchymal stem cells (IS-AD-MSC) along with bone marrow-derived hematopoietic stem cells (BM-HSC) in type 1 diabetes mellitus (T1DM). This was an institutional review board-approved prospective non-randomized open-labeled clinical trial after informed consent of 20 patients (15 males and 5 females) with T1DM for SCT, with mean disease duration of  $9 \pm 5.51$  years. Their mean age and weight were  $19.95 \pm 8.35$  years and  $49.9 \pm 14$  kg, respectively. Our study includes T1DM with positive for glutamic acid decarboxylase (GAD) antibody and history of diabetic ketoacidosis (DKA).

Generated IS-AD-MSC and BM-HSC were infused via femoral catheterization under local anesthesia into portal + thymic circulation and subcutaneous tissue with conditioning of injection rabbit anti-thymocyte globulin and Bortezomib. Patients were monitored for blood sugar, serum C-peptide, GAD antibodies, and glycosylated hemoglobin (Hb1Ac) at three monthly intervals post-therapy. Mean SC quantum infused  $99.45 \pm 22.5$  mL, with mean  $2.38 \pm 0.78 \times 10^4$  ISC/ $\mu$ L, mean CD34+ 0.57 %, and mean CD45-/90+ and CD45-/73+ were 47.22 and 24.66 %, respectively. Generated ISCs expressed transcription factors ISL-1, PAX-6, and IPF-1. Variable and sustained improvement in mean FBS, PPBS, HbA1c, and serum C-peptide was noted over a mean follow-up of  $43.94 \pm 19.8$  months with mean reduction of GAD antibody from 525.15 to 120.15 IU/mL. Mean insulin requirement decreased from 60.89 to 39.76 IU/day. There was absence of DKA after SCT. No untoward effect/morbidity/mortality was recorded from SCT. Co-infusion of IS-AD-MSC with BM-HSC offers a safe and viable therapy for T1DM.

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**Keywords** Type 1 diabetes mellitus · Insulin requirement · C-peptide · Glycosylated hemoglobin · Stem cell therapy · Mesenchymal stem cells · Hematopoietic stem cells

## Introduction

Type 1 diabetes mellitus (T1DM) is the second most common chronic disease of childhood believed to be autoimmune in nature, characterized by irreversible destruction of insulin-secreting pancreatic  $\beta$ -islet cells, requiring lifelong exogenous insulin therapy. Symptoms of the disease appear when insulin-making  $\beta$  cell mass gets reduced by approximately 90 % leading to severe insulin deficiency and hyperglycemia [1]. The

incidence of diabetes mellitus (DM) has been increasing in an epidemic-like fashion in the last two decades globally. India is expected to become the world capital of DM by year 2030 [2–4]. Sporadic cases of hematopoietic stem cell transplantation have been reported with limited success [5]. Stem cell therapy (SCT) holds great promise for cure of many diseases including T1DM. Insulin-secreting cells generated from stem cells could represent an attractive alternative [6]. Mesenchymal stem cells (MSC) have remarkable paracrine effects which can be divided into trophic (“nurturing”), immunomodulatory, anti-scarring, and chemoattractant [7]. MSC can be isolated from adipose tissue and can be differentiated into insulin-secreting cells (ISC). We present our experience of insulin replacement therapy to T1DM patients with co-infusion of *in vitro* generated ISC differentiated from adipose tissue-derived MSC along with bone marrow (BM)-derived hematopoietic stem cells (HSC).

**Aim of study:** This was the prospective, non-randomized, open-labeled, clinical trial to test the efficacy and safety of co-infusion of (autologous and allogenic) insulin-secreting adipose tissue derived mesenchymal stem cells (IS-AD-MSC) and BM-derived HSC to treat the T1DM patients.

## Methods

This clinical trial was carried out at G.R. Doshi and K.M. Mehta Institute of Kidney Diseases & Research Centre (IKDRC) - Dr. H.L. Trivedi Institute of Transplantation Sciences (ITS) between 2010 to January 2013. The institutional review board approved of consent forms and clinical trial. Our study included type 1 diabetics for more than 12 months with history of diabetic ketoacidosis (DKA) and positive for glutamic acid decarboxylase (GAD) antibody of any gender with age group of 8 to 45 years confirmed with low serum C-peptide. Exclusion criteria included positive serology for hepatitis C/hepatitis B/HIV infection, other systemic infections/disorders, malignancy, and pregnancy. Key endpoints of study were morbidity, mortality, untoward side effects from stem cell co-infusion, and changes in exogenous insulin requirements (daily dose/duration). Secondary endpoints were monitoring of GAD antibodies and serum C-peptide levels with mixed-meal tolerance test at three monthly intervals with glycosylated hemoglobin (HbA1c) following stem cell co-infusion.

Total 20 volunteers, 15 males and 5 females, were subjected for SCT. For autologous SCT, patients' own fat and BM were used after their informed written consent, labeled as group 1. For allogenic SCT, healthy non-diabetic volunteer donors from family of recipients with same compatible blood group, who were willing to donate fat and BM after their informed written consent, labeled as group 2.

**Patients' data:** Our study included total 20 volunteers with 15 males and 5 females had mean age of  $19.95 \pm 8.35$  years, mean disease duration  $9 \pm 5.51$  years, mean fasting blood sugar (FBS)  $289.55 \pm 81.5$  mg/dL, mean postprandial blood sugar (PPBS)  $354.36 \pm 70.8$  mg/dL, mean HbA1c  $11.43 \pm 2.04$  %, mean serum C-peptide  $0.13 \pm 0.18$  ng/mL, and mean insulin requirement  $60.89 \pm 21.02$  IU/day.

## Study design

**Isolation and differentiation of MSC from h-AD:** After informed consent from the patient/ donor, 10 g of adipose tissue were resected from the anterior abdominal wall under local anesthesia (LA) after making a small left paramedian incision below the umbilicus. Sutures were placed after hemostasis was secured. The adipose tissue was collected in Dulbecco's Modified Eagle's Medium (DMEM, Sigma,) (high glucose), 20 % human albumin (Reliance Life Sciences, Mumbai, India), fibroblast growth factor, 1 % sodium pyruvate, and antibiotics of penicillin, streptomycin, cefotaxime, and fluconazole (anti fungal medication). The adipose tissue was minced in collagenase type 1 (10 mg/10 mL) solution in a culture dish and placed at  $37^{\circ}\text{C}$  with a 35-RPM shaker for 1 h before centrifugation at 780 RPM 8 min. The supernates and pellets were separately cultured in proliferation medium on 100 and  $25\text{ cm}^2$  cell+ plates (Sarstedt, USA), respectively, at  $37^{\circ}\text{C}$  5 % CO<sub>2</sub> for 10 days with the medium changed every other day. On day 10 of culture in proliferation medium, cells washed with phosphate-buffered saline (1 N) were harvested by trypsinization, counted, and checked for viability and sterility. In addition to flow cytometric analysis of cells for CD 45<sup>+</sup> (per CP) and CD90<sup>-</sup> (PE)/CD73<sup>+</sup> (PE) (Beckton Dickinson, USA), the cells were stained with Giemsa. For further differentiation into insulin-expressing cells, they were placed for 3 days in differentiation medium containing DMEM (glucose, 17.5 mmol/L); 500 mL DMEM F 12:500 mL; nicotinamide 10 mmol/L; activin A 2 nmol/L; exendin 4 10 nmol/L; pentagastrin 10 nmol/L; hepatocyte growth factor 100 pmol/L; B-27, N-2 serum supplement 10 mL each; and antibiotics (penicillin [200,000U/mL], streptomycin [200,000U/mL], cefotaxime [1 g/5 mL], fluconazole [100 mg/dL]). This cocktail upregulates gene expression, nourishes the cells, and prevents further proliferation. Thereafter, the cells were subjected to isolation on Ficoll Hypaque using a density gradient. The cell pellet was diluted with an equal amount of medium and subsequently subjected to the following measurements after testing for sterility, viability, and cell counts: PAX-6, a key regulator for normal islet cell development; ISL-1, the gene upregulating insulin expression; IPF-1, regulator of  $\beta$  cell-specific gene expression, function, and for self-renewal of progenitor cells using immunofluorescence methodology as well as C-peptide and insulin measurements in the supernates of cultured cells measured by chemiluminescence. Cells were

further incubated in 6-well plates at the concentration of 5 cells/cm<sup>2</sup> without glucose and in glucose (90 mM), 5 mL and 10 mL, respectively, for 2 h and insulin and C-peptide levels were measured at the end (Fig. 1). C-peptide and insulin secretion were tested by chemiluminescence assay (Lumax, Lake Forest, CA, USA) [1].

Culture of BM: On day 10 of fat resection, 100 mL BM was aspirated under LA from the posterior superior iliac crest of the same patient/donor and subjected to in vitro expansion using DMEM:F12 (1:1) with 20 % human albumin, erythropoietin (V.H.B. Life Sciences, Inc, India), 10 µL/100 mL, G-CSF (Gennova iopharma, India), 10 µL/100 mL, Mitomycin C, 2 µL/100 mL, nonessential amino acids, 1 mL/100 mL, ascorbic acid, 10 µL/100 mL, and antibiotics in CO<sub>2</sub> incubator at 37 °C with 5 % CO<sub>2</sub> under humid conditions. No xenogenic material was used at any stage. The medium was changed every other day, and cells cultured for three passages [1].

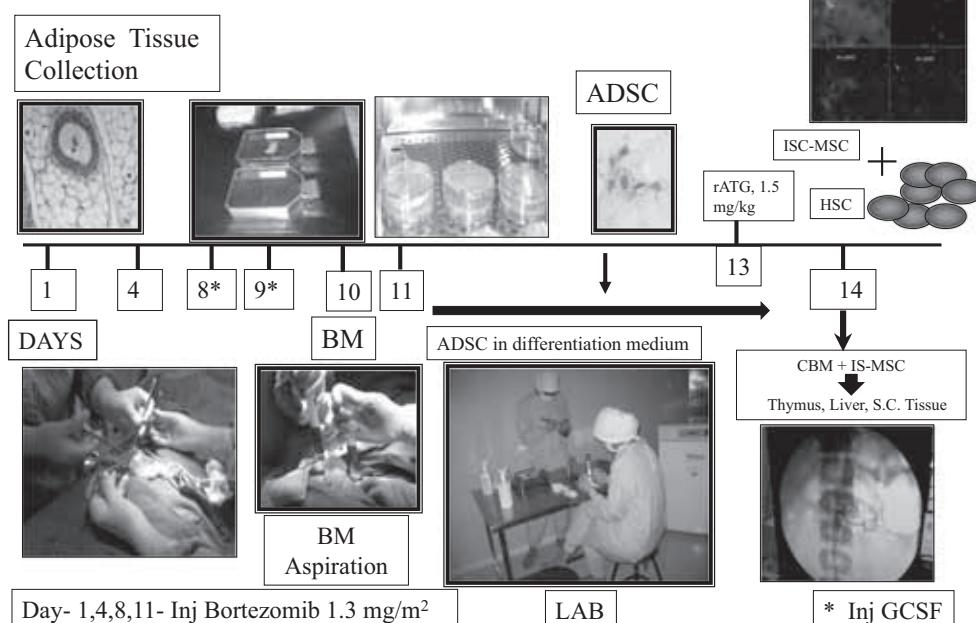
IS-AD-MSCs were generated as per above technique from 10-g adipose tissue from patient in group 1 and from donor in group 2. MSC were harvested on day 10, further differentiated into ISC on day 14, quantified and tested for sterility, viability, and insulin-secreting markers—PAX-6, IPF-1, and ISL-1 by immunofluorescence. The prepared inoculum was then mixed with generated HSC from aspirated 100 mL cultured BM on day 10 from patient in group 1 and from donor in group 2. Co-infusion of IS-AD-MSC and BM-HSC was carried out on day 14 into portal + thymic circulation via femoral catheterization under LA and into abdominal subcutaneous tissue by subcutaneous injection after conditioning with injection bortezomib, 1.3 mg/m<sup>2</sup> body surface area along with methyl prednisolone,

125 mg intravenously in 250 mL normal saline, on days 1, 4, 8 and 11 followed by rabbit anti-thymocyte globulin, 1.5 mg/kg BW on day 12.

Data pertaining to infused cells: Among all 20 patients, mean SC quantum infused was 99.45±22.5 mL with mean  $2.38\pm0.78\times10^4$  ISC/µL with mean CD34+ 0.57±0.55 %, and mean CD45-/90+ and CD45-/73+ were 47.22±13.46 and 24.66±12.89 %, respectively. Generated ISCs expressed transcription factors ISL-1, PAX-6, and IPF-1.

Patients' monitoring: Patients were monitored 4-hourly for first 2 days after infusion for blood sugar levels. FBS and PPBS levels after lunch and dinner were monitored for the next 5 days and discharged at the end of 1 week. Subsequently patients were advised to monitor FBS and PPBS weekly for the first month, fortnightly for the next 2 months, and monthly thereafter till the end of 1 year. Subsequently, they were advised to check FBS and PPBS every 3 months. Serum C-peptide and HbA1c were measured chemiluminescence assay before the infusion and 3-monthly after infusion. Glycosylated hemoglobin (HbA1c) (reference range: normal 4.2–6.2 %, good control 5.5–6.8 %, fair control 6.8–7.6 %, poor control >8 %) (Erba diagnostics, Germany) was measured every 3-monthly post-infusion. Glutamic acid decarboxylase (GAD) antibodies were measured before the infusion and 3 months after infusion by ELISA technique (normal range <10 IU/mL) (EUROIMMUN Medizinische Labordiagnostika AG, UK). Body weight and diabetic ketoacidosis (DKA) episodes were also regularly monitored. Insulin administration was made on sliding scale with an objective of maintaining FBS ≤150 mg/dL (reference

**Fig. 1** Paradigm of stem cell therapy (SCT)



range 70–110 mg/dL) and PPBS ≤200 mg/dL (reference range 80–140 mg/dL).

**Statistical analysis:** Statistical analysis was performed using SPSS version 12. Data are expressed as mean $\pm$ SD (min–max) for continuous variables. Continuous variables were compared using Wilcoxon signed rank test.  $p<0.05$  was considered to be statistically significant. Insulin requirement, HbA1c, serum C-peptide levels, FBS, and PPBS were monitored.

## Results

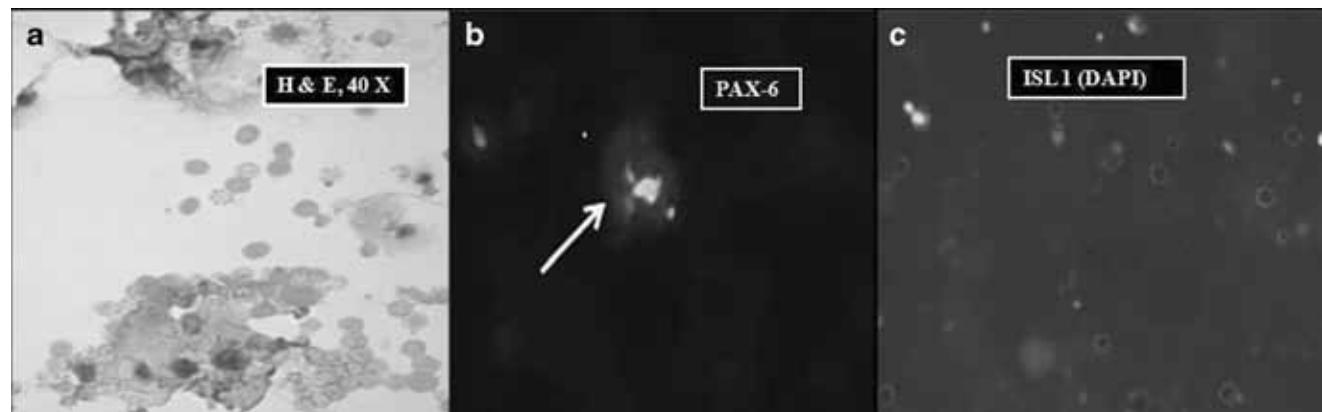
Harvested MSCs were further differentiated into ISC which confirmed with insulin-secreting markers—PAX-6, IPF-1, and ISL-1 by immunofluorescence (Fig. 2). Mean insulin-secreting cell count was  $2.38\pm 0.78 \times 10^4/\mu\text{L}$ . No untoward effect, morbidity, or mortality was recorded in this study. Variable and sustained improvement in mean FBS, PPBS, HbA1c, and serum C-peptide noted over a mean follow-up of  $43.94\pm 19.8$  months (Fig. 3). Mean GAD antibody has decreased from  $525.15\pm 682.4$  to  $120.1\pm 217$  IU/mL. Mean insulin requirement decreased from 60.89 to 39.76 IU/day (Table 1). No functional correlation was observed between exogenous insulin requirement with C-peptide levels and GAD antibody levels. There was an impressive absence of DKA episodes in all of them with improved subjective energy levels.

## Discussion

Replacement of ISCs represents an almost ideal treatment for patients with T1DM as an alternate to islet cell transplantation. Potential therapy for T1DM needs to address insulin replacement and immune dysregulation arising in these patients.

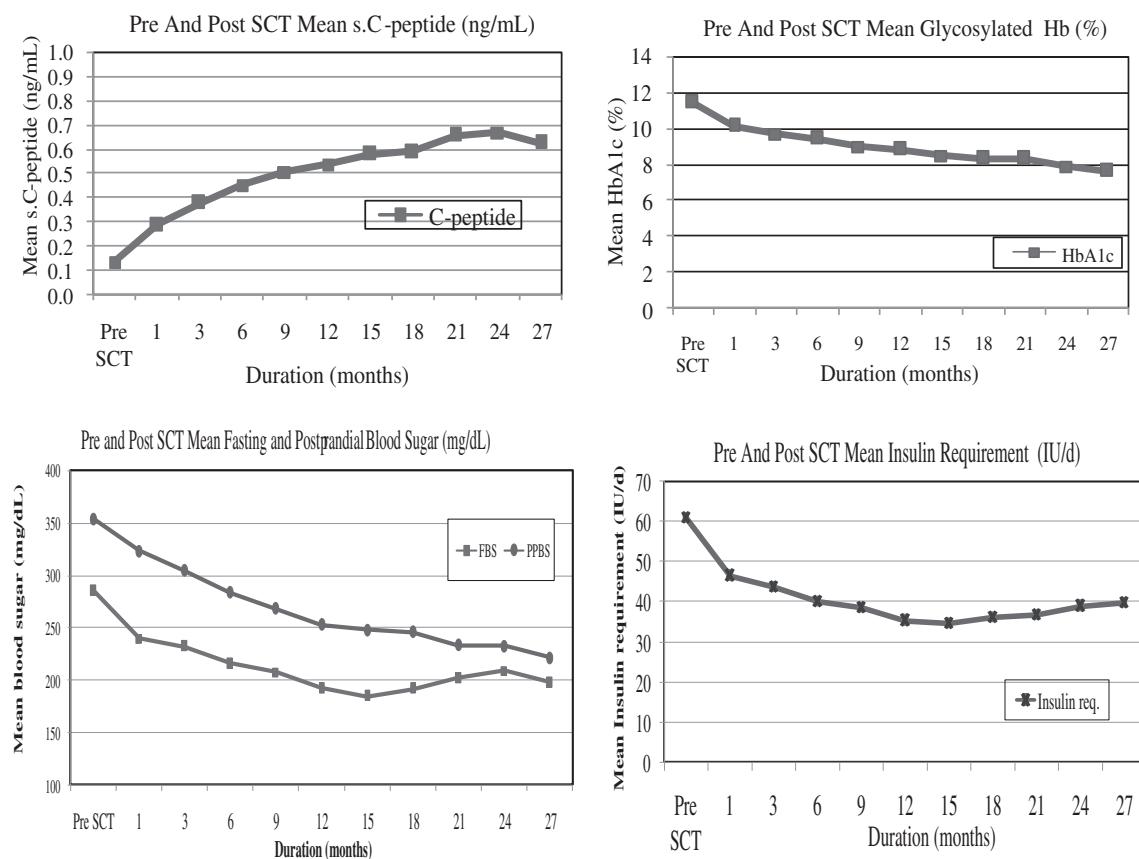
Studies till now have demonstrated the feasibility of generating insulin-producing cells obtained from progenitor cells of various sources, including the pancreas [8, 9], liver [10], and intestinal epithelium [11], as well as the pluripotent embryonic SCs of mouse and human origin [12, 13]. The most effective protocols till date have produced cells that express insulin and have molecular characteristics that closely resemble bonafide ISCs; however, these cells are often unresponsive to glucose, which is also a most vital characteristic concern which needs to be solved before finding a definite clinical application.

Generation of ISCs from SCs represents an attractive alternate. Among adult SCs, MSCs appear to have a particular developmental plasticity ex vivo that includes their ability to adopt a pancreatic endocrine phenotype [14]. Transplantation of islets of Langerhans was shown to be successful in experienced centers, but due to shortage of organs and lifelong immunosuppression, this therapy can be offered to a very limited number of patients only [15, 16]. In animal models of T1DM, MSC showed beneficial effects in glycemic control, either isolated [17] or combined to HSC [18]. Among SCs, MSCs have several advantages for therapeutic use such as ability to migrate to the sites of tissue injury and strong immunosuppressive effects [19–22] in addition to riding over the hurdles involved in application of human embryonic SCs. In 2008, a group of researchers started studies in humans with T1DM using BM-derived MSC [23]. Their protocol included BM aspiration under general anesthesia from first-degree relatives for collection of MSC. These cells were sent to a laboratory to be stimulated to proliferate for a month and were later infused into patients through a gelatinous solution of approximately 100 mL, without use of chemotherapy. The patients were hospitalized for 1 day. Repeat infusion was followed after a month. So far, they are not yet sure how many infusions will be necessary. Two patients have been recruited in their protocol; however, follow-up data has been published as yet.



**Fig. 2** In image **a**, Harvested mesenchymal stem cells with hematoxylin and eosin stain showing centrally placed round nucleus with clear nuclear margin and surrounding fine granular eosinophilic cytoplasm under

microscope, and in images **b** and **c**, generated insulin-secreting cells expressed transcription factors ISL-1 and PAX-6 seen with indirect immunofluorescent study and DAPI (4,6-diamidino-2-phenylindole)



**Fig. 3** Stem cell Therapy (SCT) response to glycosylated hemoglobin, serum C-peptide, daily insulin requirement, and fasting postprandial blood sugar status

**Table 1** Statistical analysis for insulin requirement, Hb1Ac, serum C-peptide levels, FBS and PPBS over 27-months follow-up of total 20 patients treated with stem cell therapy

N=20	Insulin req. (IU/day)	HbA1c (%)	S.C-peptide (ng/mL)	FBS (mg/dL)	PPBS (mg/dL)
Pre SCT	60.9±21.0	11.4±13.0	0.13±0.18	289.5±81.5	354.3±70.8
Post SCT	46.5±21.5	10.1±1.8	0.28±0.28	239.5±74.6	323.0±79.6
1 month					
Post SCT	43.6±19.8	9.7±1.9	0.37±0.34	231.9±71.9	304.5±81.1
3 months					
Post SCT	40.1±18.8	9.5±1.6	0.45±0.36	215.6±68.5	283.6±80.6
6 months					
Post SCT	38.5±14.1	8.9±1.6	0.50±0.35	207.7±68.8	268.1±71.9
9 months					
Post SCT	35.3±12.8	8.8±1.5	0.53±0.31	191.7±68.3	253.0±69.3
12 months					
Post SCT	34.6±10.8	8.5±1.5	0.57±0.30	184.2±58.1	247.9±65.2
15 months					
Post SCT	36.1±9.1	8.3±1.2	0.58±0.34	191.2±58.5	245.7±56.3
18 months					
Post SCT	36.8±9.7	8.3±0.9	0.65±0.31	201.6±55.8	233.2±55.4
21 months					
Post SCT	39.0±11.4	7.9±1.0	0.65±0.36	209.1±59.2	232.5±51.4
24 months					
Post SCT 27 months	39.8±13.2	7.6±1.4	0.62±0.36	197.4±38.2	221.4±52.2

SCT stem cell therapy, HbA1c glycosylated hemoglobin, FBS fasting blood sugar, PPBS postprandial blood sugar; N=20, patients of type 1 DM

Immune-modulatory properties of MSC, presence of various markers for multipotent pancreatic stem cells, and enhanced expression of various pancreatic genes during the differentiation of MSC [24, 25] stimulated us to study the effect of ISCs differentiated from MSC in T1DM. MSCs are able to serve as a cellular vehicle for the expression of human insulin gene and are expected to become promising therapeutic agents in the treatment of the complications of DM-like cardiac function and treatment of diabetic cardiomyopathy/nephropathy/polyneuropathy and wounds in diabetic patients [26]. We have generated *in vitro* MSC from human adipose tissue which qualify the definition standardized by the Mesenchymal and Tissue Stem Cell Committee of the International Society for cellular therapy. We further differentiated them to ISCs under defined culture conditions phenotypically identical to pancreatic  $\beta$  cells. These cells expressed transcription factors IPF-1, PAX-6, and ISL-1. All three are central controlling genes capable of reprogramming non-pancreatic cells to surrogate  $\beta$  cell functions. Again, our technique is a shortcut to reprogramming non-pancreatic cells as compared to vector-based gene transfer techniques [1]. The HSCs were used along with IS-ADMSC because HSC infusion with conditioning is believed to create active and passive tolerance by clonal deletion/T cell suppression [27]. Hence, we decided to explore this protocol which has already given sustained benefits without any adverse effects and decided to infuse the cells in thymic circulation to achieve central tolerance [28] and portal circulation since liver is the most tolerogenic organ [29]. Subcutaneous tissue being an immunologically privileged site, we decided to inject part of the cells in abdominal subcutaneous tissue, so that it will serve as a “backup reservoir” for insulin supply [30].

As compared to all the above studies, the present study on 20 T1DM patients of generating and infusing ISC from ADMSC, along with BM-HSC under self-designed culture conditions containing high concentration of glucose and the addition of insulin-stimulating factors is encouraging. *In vitro* functionality of these cells was confirmed by insulin production and release in a glucose-responsive manner. This was achieved without genetic modification. The major advantage of this study is that it throws light on availability of source as an adipose tissue rather than other sources and alternative medications to control T1DM. Our results support the contention that combination of these three transcription factors represents the establishment of ectopic mechanisms to secrete insulin. These exciting results raise intriguing questions; whether this differentiation involves epigenetic reprogramming or nuclear content of the MSC has become permissive to allow activation of this transcription program for  $\beta$  cell function. Whatever may be the reason, this strategy has worked even in distantly related cells of origin. However, the questions that remain unanswered are the following: clinical dilemma involving the issue of autoimmunity; will the

immune response to infused cells destroy the infused insulin-producing cells with longer time span? How much is the dose of cells required to achieve complete cure T1DM? Should it require more potent or supporting cells like regulatory T cells? We believe the results demonstrated in this study provide evidence supporting the notion that differentiation of autologous/allogenic human adipose tissue-derived MSC to ISCs may represent a viable therapeutic option for T1DM without use of any immunosuppression post SCT. This study opens new avenues for managing different diabetic patients.

**Conclusion:** From our experience, we conclude that this is the first step towards the use of IS-AD-MSC as a cell-based treatment for T1DM, may be the alternate to islet cell transplantation. This is the first report of successfully treated T1DM with history DKA and positive GAD antibody with co-infusion of IS-AD-MSC and BM-HSC of either autologous or allogenic sources, with relatively simple and easy technique, and offers a safe and viable approach.

*BM* bone marrow, *BM-HSC* bone marrow-derived hematopoietic stem cells, *CBM* cultured bone marrow, *DKA* diabetic ketoacidosis, *FBS* fasting blood sugar levels, *GAD* glutamic acid decarboxylase, *HbA1c* glycosylated hemoglobin, *IPF-1* insulin promoter factor 1, *IS-AD-MSC* insulin-secreting adipose tissue-derived mesenchymal stem cells, *ISC* insulin-secreting cell, *ISL-1* islet-1, *LA* local anesthesia, *MSC* mesenchymal stem cells, *PPBS* postprandial blood sugar, *PAX-6* paired box gene 6, *SCs* stem cells, *T1DM* type 1 diabetes mellitus

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**Conflict of interest** The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

# A retrospective study of maternal and neonatal outcomes in overweight and obese women with gestational diabetes mellitus

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**Abstract** In pregnant women, obesity is a risk factor for multiple adverse pregnancy outcomes, including gestational diabetes mellitus (GDM), preeclampsia, and preterm birth. The aim of this study was to determine the effects of pre-pregnancy body mass index (BMI) on maternal and neonatal outcomes in women with GDM. A retrospective study of 5010 patients with GDM in 11 provinces in China was performed in 2011. Participants were divided into three groups based on BMI as follows: a normal weight group ( $BMI\ 18.5\text{--}23.9\ kg/m^2$ ), an overweight group ( $BMI\ 24\text{--}27.9\ kg/m^2$ ), and an obese group ( $BMI\ \geq 28.0\ kg/m^2$ ). Maternal baseline characteristics and pregnancy and neonatal outcomes were compared between the groups. Multiple logistic regression analysis was used to explore the relationships between BMI and the risk of adverse outcomes. Of the 5010 GDM patients, 2879 subjects were from north China and 2131 were from south China. Women in the normal weight group gained more weight during pregnancy compared with the overweight and obese GDM patients. Women in the

overweight and obese groups had increased odds of hypertension during pregnancy (adjusted odds ratio (AOR)=1.50, 95 % confidence interval (CI)=1.31–1.76 and AOR=2.12, 95 % CI=1.84–3.16). The AORs for macrosomia in the overweight and obese groups were 1.46 (95 % CI=1.16–1.69) and 1.94 (95 % CI=1.31–2.98), respectively. The relative risk of delivering a baby with an Apgar score  $<7$  at 5 min was significantly higher in women who were obese (AOR=2.11, 95 % CI=1.26–2.85) before pregnancy compared with normal weight women. Compared with the normal weight subjects, the incidence of cesarean section and emergency cesarean section among overweight and obese women with GDM was significantly higher ( $P<0.001$ ). Overall, overweight and obese women with GDM have an increased risk of adverse outcomes, including hypertension during pregnancy, macrosomic infants, infants with low Apgar scores, and the need for an emergency cesarean section. More attention should be paid to GDM women who are obese because they are at risk for multiple adverse outcomes.

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**Keywords** Gestational diabetes mellitus · Obesity · Pregnancy complications · Body mass index

## Introduction

Gestational diabetes mellitus (GDM) is a common metabolic complication during pregnancy. Characterized by glucose intolerance, GDM affects approximately 4.3 % of pregnancies in China [1]. As a major cause of both maternal and neonatal mortality and morbidity, GDM poses a greater risk for macrosomia, cesarean delivery, newborn congenital malformations, and stillbirth. Compared with those who have a normal pregnancy, women with a history of GDM have a significantly higher risk of metabolic syndrome [2]. Furthermore, a history of GDM is a risk factor for type 2 diabetes and

cardiovascular diseases [3], and children born to mothers with prior GDM have a higher risk of overweight and obesity in the future [4].

It has been reported that multiple factors, such as a higher pre-pregnancy body mass index (BMI), abdominal circumference, fasting glycemia in the first trimester of pregnancy, and the presence of polycystic ovary syndrome, are strongly associated with an elevated GDM risk [5]. As a preventable and reversible contributor to GDM, obesity has been one of the most important health problems worldwide. It is reported that overweight is a major threat to a successful pregnancy outcome, leading to preeclampsia, cesarean delivery, cephalopelvic disproportion, and macrosomia [6]. Additionally, gestational weight gain is an important factor in both maternal and infant outcomes. High weight gain ( $>18$  kg) during pregnancy is associated with preeclampsia and cesarean delivery [7]. Many studies have already focused on the association between obesity and GDM. Evidence from Singh et al. suggested that the risk of GDM increased with an increasing BMI across all weight categories. The odds ratio (OR) of incident GDM was 1.08 (95 % confidence interval (CI) 1.08–1.09) for each 1  $\text{kg}/\text{m}^2$  increase in BMI and 1.48 (95 % CI 1.45–1.51) for each 5  $\text{kg}/\text{m}^2$  increase, indicating that pre-pregnancy BMI plays an important role in GDM risk [8]. Due in large part to the obesity epidemic, GDM is becoming increasingly common worldwide.

Although a few studies have investigated the association between obesity and GDM, the majority of these studies was limited by small sample sizes and may not be applicable to the Chinese population [9, 10]. The objective of the present survey was to investigate the pregnancy outcomes among overweight and obese GDM patients in China.

## Materials and methods

This was a hospital-based, retrospective cohort study of women whose pregnancies were complicated by GDM. This study included GDM women who gave birth to a singleton in 11 provinces in China. Data were available from medical records in 2011. Patients were selected from 36 different hospitals in mainland China. One tertiary- or secondary-level hospital was randomly selected in each province. The study was conducted with the permission of the institutional review board at each hospital and was approved by the ethics committees of the medical institutions involved.

All pregnant women with singleton pregnancies diagnosed with GDM in our selected hospitals with a pre-pregnancy  $\text{BMI} \geq 18.5 \text{ kg}/\text{m}^2$  were identified for this retrospective study. The pre-pregnancy BMI was obtained from a self-reported medical record. Exclusion criteria included maternal pre-pregnancy chronic disease, including chronic hypertension,

history of diabetes, and multiple gestations. Finally, 5010 GDM women were included in the present analysis.

GDM was defined according to the criteria recommended by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) [11]. GDM was diagnosed when any of the following values were met in the 75-g oral glucose tolerance test (OGTT) performed in gestational week 24–28: fasting blood glucose 5.1  $\text{mmol}/\text{l}$ , 1-h blood glucose 10.0  $\text{mmol}/\text{l}$ , or 2-h blood glucose 8.5  $\text{mmol}/\text{l}$ . The treatments for GDM included dietary consultation and insulin treatment wherever necessary.

We classified women into three groups depending on their pre-pregnancy BMI based on recommendations by the Group of China Obesity Task Force of the Chinese Ministry of Health as follows: normal weight (BMI between 18.5 and 23.9  $\text{kg}/\text{m}^2$ ), overweight (BMI between 24.0 and 27.9  $\text{kg}/\text{m}^2$ ), and obese ( $\text{BMI} \geq 28.0 \text{ kg}/\text{m}^2$ ).

Baseline characteristics were compared between different groups. Assisted reproductive technology (ART) pregnancies include pregnancies achieved from in vitro fertilization (IVF) or intracytoplasmic sperm injection/embryo transfer (ICSI/ET). We compared the pregnancy outcomes between groups. Gestational hypertension was defined when the systolic blood pressure was  $\geq 140 \text{ mmHg}$  or diastolic blood pressure  $\geq 90 \text{ mmHg}$ , measured at two different intervals with woman at rest for more than 15 min. Preterm delivery was defined as delivery of a liveborn infant before 37 gestational weeks [12]. Postpartum hemorrhage was defined as blood loss greater than 500 mL in the first 24 h after delivery. Macrosomia was defined as a birth weight of at least 4000 g.

For the statistical analysis, continuous variables were expressed as the means  $\pm$  standard deviation as determined by one-way analysis of variance (ANOVA). The chi-square test, Fisher's exact test, and one-way ANOVA were used to compare variables between different groups. Multivariate logistic regression analysis was performed to estimate the association between pre-pregnancy BMI and adverse outcomes. Adjusted odds ratios (AORs) and 95 % CIs were calculated for all risk factors. Additionally, the outcomes were adjusted for maternal age, parity, gestation weeks, and smoking status.  $P < 0.05$  was considered significant. All statistical analyses were performed using SPSS version 17.0.

## Results

A total of 5010 women with GDM participated in the study. Participants were selected from 36 hospitals in 11 Chinese provinces. We divided the hospitals into two sections based on their location. The rate of obesity was 13.9 % in north China, while it was 7.1 % in south China. The regional distribution of women according to their pre-pregnancy BMI is summarized in Table 1.

**Table 1** Regional distribution of pregnant women with GDM in China

Region	Hospital	GDM	Normal weight	Overweight	Obese
North China	22	2879	1985/2879 (68.9 %)	493/2879 (17.1 %)	401/2879 (13.9 %)
South China	17	2131	1717/2131 (80.6 %)	262/2131 (12.3 %)	152/2131 (7.1 %)
Total	39	5010	3702	755	553

The baseline characteristics of the study population are listed in Table 2. A total of 3702 GDM patients had a normal pre-pregnancy BMI, 755 were overweight, and 553 were obese. The mean BMIs in the three groups were  $20.9 \pm 1.9$ ,  $25.9 \pm 1.4$ , and  $30.6 \pm 2.1 \text{ kg/m}^2$ , respectively. There were no significant differences in age, gestational weeks, and alcohol consumption between the different groups. Compared with the overweight and obese women, the normal weight women were more likely to gain more weight during pregnancy ( $P < 0.001$ ). In addition, the percentage of smokers in the normal weight group was higher than that in the other groups ( $P < 0.001$ ).

Table 3 shows the maternal and neonatal outcomes in different groups. The rates of premature rupture of membranes (PROM), preterm labor, placental abruption, postpartum hemorrhage (PPH), and neonatal malformation were not significantly different between the three groups. An increased risk of hypertension during pregnancy (HDP) was observed in overweight and obese GDM patients compared with normal weight patients. The AORs in the overweight and obese groups were 1.50 and 2.12, respectively, compared with the normal weight group. ORs were adjusted for maternal age, gravidity, gestational week, and smoking status. The neonatal outcomes were also different between the groups. Overweight GDM mothers were more likely to deliver a macrosomic infant (AOR=1.46, 95 % CI=1.16–1.69), and the AOR for macrosomia in the obese group was even higher (AOR=1.94, 95 % CI=1.31–2.98). The relative risk of delivering a baby with an Apgar score  $<7$  at 5 min was markedly higher in women who were obese (AOR=2.11, 95 % CI=1.26–2.85) prior to pregnancy.

The modes of pregnancy and delivery in GDM women in the different groups are compared and summarized in Table 4. There were no significant differences between the three groups with regard to the mode of pregnancy, including

spontaneous pregnancy and ART pregnancy. However, at delivery, the rate of vaginal delivery was significantly decreased in both the overweight and obese groups. The rate of cesarean delivery was higher in the overweight group than in the normal weight group (65.0 vs. 48.8 %), and the cesarean delivery rate was highest in the obese group (74.5 %). Furthermore, a significantly increased risk of emergency cesarean section was observed in the overweight and obese groups compared with the normal weight group.

## Discussion

As previously reported, overweight and obesity at the beginning of pregnancy can lead to adverse pregnancy outcomes, including GDM, gestational hypertension, preeclampsia, induction of labor, prolonged second stage of labor, postpartum hemorrhage, and neonatal macrosomia [13, 14]. Our results suggest that a high pre-pregnancy BMI increased the risk for gestational hypertension, fetal macrosomia, delivering a baby with an Apgar score  $<7$  at 5 min, and cesarean section after adjustment for maternal age, gravidity, gestational age, and smoking status in GDM women. These results demonstrate that obesity is not only a risk factor for GDM but is also a risk factor for adverse pregnancy outcomes induced by GDM.

As one of the most common medical complications of pregnancy, GDM is characterized by glucose intolerance due to an insufficiency of insulin secretion to meet the increased requirements during pregnancy. Previously published studies suggested that insulin secretion and action in GDM were directly affected by adipocyte-derived factors such as leptin and adiponectin [15, 16]. A meta-analysis by Chu et al. involving 20 studies revealed that compared with normal weight pregnant women, overweight, obese, and severely obese women had ORs of developing GDM of 2.14, 3.56, and 8.56,

**Table 2** Demographic characteristics in different groups

Variable	Normal weight (n=3702)	Overweight (n=755)	Obese (n=553)	P value
Age (years)	$30.5 \pm 4.8$	$31.1 \pm 5.7$	$29.4 \pm 4.9$	0.201
Pre-pregnancy BMI ( $\text{kg}/\text{m}^2$ )	$20.9 \pm 1.9$	$25.9 \pm 1.4$	$30.6 \pm 2.1$	$P < 0.001$
Weight gain (kg)	$14.8 \pm 6.1$	$12.9 \pm 4.7$	$12.2 \pm 5.4$	$P < 0.001$
Gestational week	$37.5 \pm 7.3$	$37.2 \pm 4.7$	$38.6 \pm 1.9$	0.301
Smoking	0.4 %	0.3 %	0.2 %	$P < 0.001$
Alcohol consumption	0.4 %	0.3 %	0.3 %	0.093

**Table 3** Pregnancy and neonatal outcomes in the three groups

Incidence	Normal weight (n=3702)	Overweight (n=755)	Obese (n=553)		
	n (%)	n (%)	Adjusted OR (95 % CI)	n (%)	Adjusted OR (95 % CI)
<b>Pregnancy outcome</b>					
HDP	312 (8.4 %)	114 (15.1 %)	1.50 (1.31–1.76) <sup>#</sup>	166 (30.0 %)	2.12 (1.84–3.16) <sup>#</sup>
PROM	711 (19.2 %)	126 (16.7 %)	1.02 (0.63–1.54)	88 (15.9 %)	0.94 (0.91–1.28)
Preterm labor	270 (7.3 %)	53 (7.0 %)	0.86 (0.49–1.38)	34 (6.1 %)	0.86 (0.48–1.31)
Placental abruption	60 (1.6 %)	12 (1.6 %)	1.12 (0.58–1.40)	9 (1.6 %)	1.04 (0.75–2.89)
PPH	225 (6.1 %)	48 (6.4 %)	0.96 (0.69–1.81)	38 (6.8 %)	1.23 (0.97–1.86)
<b>Newborn outcome</b>					
Macrosomia	349 (9.4 %)	116 (15.4 %)	1.46 (1.16–1.69) <sup>#</sup>	121 (21.9 %)	1.94 (1.31–2.98) <sup>#</sup>
5 min Apgar score <7	6 (0.2 %)	3 (0.4 %)	1.17 (0.82–1.65)	4 (0.7 %)	2.11 (1.26–2.85)*
Malformation	32 (0.9 %)	6 (0.8 %)	0.91 (0.72–1.53)	5 (0.9 %)	1.04 (0.42–2.01)

Adjusted for maternal age, gravidity, gestational age, and smoking status

HDP hypertension during pregnancy, PROM premature rupture of membranes, PPH postpartum hemorrhage

\* $P<0.05$ ; <sup>#</sup>  $P<0.01$

respectively [17]. Furthermore, in another study, Ogonowski et al. demonstrated that with an increasing pre-pregnancy BMI, the risk for GDM increases not only in overweight but also in normal weight women [18]. These findings demonstrated that BMI and GDM are closely related.

Because obesity and GDM are the two risk factors for adverse pregnancy outcomes, we investigated the influence of different BMI categories on pregnancy outcomes in GDM patients. Our results showed that there was an increased risk of gestational hypertension in the overweight (OR=1.50, 95 % CI=1.31–1.76) and obese (OR=2.12, 95 % CI=1.84–3.16) groups compared with the normal weight group, which was in accordance with the conclusion drawn by Zhang et al., who found that the incidence of preeclampsia among obese GDM women was three- and twofold higher than those in normal weight and overweight GDM women, respectively [19]. However, we cannot confirm their findings that the risk of PROM in the obese group was 1.4- and 1.6-fold greater than those in the normal weight and overweight groups,

respectively. The reason for this difference maybe that we adjusted for other important covariates to reduce interference in the results. In addition, the weight gain in normal weight women was markedly greater than that in overweight and obese GDM women, which may also have contributed to the difference in the results. Bodnar et al. explained the mechanisms underlying the effects of obesity on preeclampsia risk as an elevated inflammatory response and high triglyceride levels [20]. However, no significant difference in the incidence of preterm labor, placental abruption, PROM, and postpartum hemorrhage was observed between the different groups.

Neonatal outcomes can also be influenced by a high pre-pregnancy BMI. As is already known, women with either GDM or obesity have an increased incidence of macrosomic neonates. Our results confirmed that the incidence was even higher among overweight and obese GDM women compared with normal weight GDM women. According to a retrospective study of 10,468 European children, fetal macrosomia is

**Table 4** Pre-pregnancy BMI and the mode of pregnancy and delivery

	Normal weight (n=3702)	Overweight (n=755)	Obese (n=553)	P value
<b>Mode of pregnancy</b>				
Spontaneous pregnancy	3608 (97.5 %)	737 (97.6 %)	539 (97.5 %)	
ART pregnancy	94 (2.5 %)	18 (2.4 %)	14 (2.5 %)	0.184
<b>Mode of delivery</b>				
Vaginal delivery	1896 (51.2 %)	256 (33.9 %)	141 (25.3 %)	
Cesarean delivery	1806 (48.8 %)	499 (65.0 %)	412 (74.5 %)	<0.001
Elective	1358 (75.2 %)	321 (64.3 %)	208 (50.5 %)	
Emergency	448 (24.8 %)	178 (35.7 %)	204 (49.5 %)	<0.001

independently associated with the development of overweight/obesity during childhood [21]. Despite this result, we found that the incidence of a low Apgar score was increased in the obese group compared with the other groups, demonstrating an adverse outcome in infants from obese GDM mothers. Gesche et al. also reported that birth weight is related to maternal BMI in obese women and that adherence to gestational weight gain recommendations does not seem to prevent the incidence of increased birth weight [22]. Moreover, increased placental inflammation due to obesity may directly affect neonatal development through alterations in nutrient transport and has a negative impact on the neonatal immune system [23].

We also studied the relationship between pre-pregnancy BMI and the mode of pregnancy and delivery. Women in the overweight and obese groups tended to have a higher rate of ART pregnancies, but this difference was not significant. Generally, obese women experience longer times to conception, even if they are young and cycling regularly. This finding could be attributed to the altered ovarian follicular environment and increased metabolite, C-reactive protein, and androgen activity levels in these women [24]. Based on our survey, the rates of cesarean section in normal weight, overweight, and obese GDM patients were 48.8, 65.0, and 74.5 %, respectively. Compared with normal weight women, the emergency cesarean section rate was 1.4- and 2.0-fold higher in the overweight and obese groups, respectively. Excess abdominal adipose tissue can obstruct the progression of labor mechanically, which in turn blocks fetal circulation and may lead to fetal distress and emergency cesarean section. Cesarean section can increase the risk of maternal and fetal mortality compared with vaginal delivery. All these factors contribute to a worse outcome in obese GDM women.

There are several limitations to this study. First, this is a retrospective study. We collected data on 5010 GDM patients nationwide, but we were limited by what information was available in the medical records. Some clinical data were not included in our survey form, and different results may have been obtained if we had the missing data. Patients who participated in this survey were from tertiary- or secondary-level hospitals, which may have contributed to selection bias. Another limitation is related to the different methods of data capture. The glucose level in GDM patients was not available; thus, the confounding role of this level in each patient could not be assessed.

## Conclusions

This study showed a significant association between pre-pregnancy BMI and an increased risk of adverse pregnancy and neonatal outcomes, such as gestational hypertension, fetal macrosomia, and emergency cesarean section, in Chinese

women. Weight control should be emphasized prior to pregnancy to achieve a better pregnancy outcome, especially for women with GDM.

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**Conflict of interest** The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

# Noninvasive blood glucose measurement utilizing a newly designed system based on modulated ultrasound and infrared light

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**Abstract** The approach of a new noninvasive innovation for blood glucose measurement will reform management of diabetes alongside expanded patient compliance, decline load on therapeutic crisis, and diabetes associated complications. In this present work, we represent a modulated ultrasound and infrared technique-based noninvasive system for blood glucose measurement over human subjects. The oral glucose tolerance test- and random blood glucose level test-based clinical study was performed over human subjects to measure the performance of our indigenously designed system. The accuracy was examined by pairing and comparing our noninvasive predicted data with the invasive reference data. The oral glucose tolerance test and random blood glucose test produced a total of 180 and 30 paired glucose values (noninvasive vs. invasive), respectively. The root mean squared error between the noninvasive and invasive glucose value for oral glucose tolerance test and random blood glucose measurement was 23.76 mg/dl and 28.20 mg/dl, respectively. The Pearson correlation coefficient between the noninvasive and invasive glucose value for both the tests was 0.76 and 0.85, respectively. Similarly, the mean absolute error for both the tests was 15.92 mg/dl and 17.76 mg/dl, respectively. Further, the Clarke Error Grid analysis depicts that both the tests result

occupy medically accurate and acceptable zones A and B, respectively (oral glucose tolerance test: zone A=78.33 %; zone B=21.66 % and random blood glucose measurement: zone A=83.33 %; zone B=16.66 %). Hence, the present study direct towards the potentiality of our technique for noninvasive blood glucose measurement. The instrument was medically safe and well tolerated.

**Keywords** Oral glucose tolerance test · Random blood glucose level · Ultrasound · Infrared light · Noninvasive technique

## Introduction

Diabetes has progressed as an important healthcare endemic of the contemporary world. Globally, the aggregate number of individuals with diabetes ascends from 382 million in 2013 to 592 million in 2030. Similarly, in our country (India), the total number of diabetic population increase from 65.1 million in 2013 to 109 million in 2030. Overall, the numbers of diabetics are expanding at an enormous alarming pace [1].

The diabetic subject needs to monitor blood glucose level (BGL) regularly for four to five times per day for circumventing the terminal stage medical complexities and expenses. The invasive device-based blood glucose monitoring comprises painful procedures, mental agony, and infection liabilities. For all these reasons, at present, a new noninvasive technique for blood glucose measurement is extremely demanding [2–6].

In perspective of addressing the requirement for this necessity, the present work's objective is to validate our indigenously developed noninvasive technique for blood glucose measurement in the human subjects.

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## Literature review and our concept for noninvasive blood glucose measurement

Zhu et al. (2013) and Zhu et al. (2010) demonstrated a new hybridized technique of utilizing an ultrasound-modulated optical technique for noninvasive blood glucose measurements. Measurement of modulation depth through ultrasound-modulated scattered light produces significant *in vitro* results. However, their technique lacks in *in vivo* experiments [7, 8].

They had applied ultrasound with light modulation techniques [7, 8], but in our application, the modulated ultrasound produces vibration and the infrared light quantity, those molecule specific vibrations for noninvasive measurement of blood glucose levels in the human subjects.

When the amplitude modulating ultrasonic waves enters inside the measurement site (human finger), the blood constituent molecules undergoes vibration due to the influence of the amplitude-modulated ultrasonic waves. The pressure of the ultrasound waves generates those changes. The intrinsic properties of these molecules present in the blood medium influences the compressibility factor. The shape and size of the molecules present in the blood medium (segment) play an important role. The magnitude of influence over larger molecules is more as compared to the smaller ones [9–11].

Hence, the vibration produced in the blood medium (segment) depends on (i) its spatial arrangement, (ii) intrinsic property of the molecules and medium, and (iii) strength and frequency of the amplitude-modulated ultrasonic waves, respectively [9–11].

The pressure amplitude of the ultrasonic standing wave has maximum and minimum values twofold above the distance of a unit wavelength. Within the propagating blood tissue medium (segment), discontinuities such as blood molecules (glucose), cells achieve location-specific ultrasonic potential energy due to their presence in the respective ultrasonic zone. The suspended blood molecules (glucose) start to move and accumulate near to the zone of least ultrasonic potential energy. For blood molecules (glucose), these concentrated zones are commonly near to the pressure nodes, distanced from each other by half a wavelength space [9–11].

Now, when the molecular diameter is smaller than the wavelength of propagating ultrasound inside the measurement site, the main force of radiation ( $F_r$ ) acting on the molecular volume ( $V_c$ ), positioned by path of distance ( $z$ ) from the pressure node, is obtained from the gradient of the molecular ultrasonic potential energy [9–11], and mathematically expressed as:

$$F_r = -\left[\frac{\pi p_0^2 V_c \beta_w}{(2\lambda)}\right] \cdot \Phi(\beta, \rho) \cdot \sin(4\pi z/\lambda) \quad (1)$$

In this present work,  $P_0$  stands for peak amplitude of the ultrasonic pressure. ( $\lambda$ ) denotes ultrasound wavelength in the

suspending segment.  $\beta_w$  signifies compressibility of the suspending segment [9–11] and mathematically expressed as:

$$\Phi(\beta, \rho) = \left[ \frac{5\rho_c - 2\rho_w}{2\rho_c + \rho_w} - \left( \frac{\beta_c}{\beta_w} \right) \right] \quad (2)$$

where  $\beta_c$  stands for compressibility of the molecules. Notations  $\rho_c$  and  $\rho_w$  represent the respective densities of the molecules and the suspending segments, respectively [9–11].

The well-known Lambert-Beer was law applied here to measure the specific absorption ( $A$ ) property of the glucose molecule at a definite light wave number ( $\nu$ ) and mathematically expressed as:

$$A(\nu) = -\log I(\nu)/I_0(\nu) \quad (3)$$

In this present work, the  $I_0$  denotes the background intensity and  $I$  represents the specific light intensity at the particular wave number ( $\nu$ ) of actual measurements [11].

Hence, as per Urban et al. (2010) and Silva et al. (2007), we achieve the benefit of beam forming at ultrasonic frequency for localizing the radiating force energy towards the particular measurement site (human finger). It initiates the vibration phenomenon at lower frequency such that the displacements are large enough for quantification with the infrared technique, respectively [12–14].

In this present work, the signal processing toolbox of MATLAB performs observed signal analysis in Fast Fourier Transform (FFT) domain to extract blood glucose level-related embedded information. The peak voltage amplitude variations in FFT domain serves as the functional indicator for measuring actual blood glucose level in study subjects. Hence, this principle aspect forms the basis of our noninvasive blood glucose measurement.

## Our noninvasive technique-based system descriptions

Initially, it includes the description regarding ultrasound transducer and light wavelength selection criterions followed by the explanation of the indigenously designed system.

### Ultrasound frequency selection

Ultrasonic transducers have found numerous applications in household appliances, medical fields, industries, and oceanography [15, 16]. In the biomedical field, the 40-kHz piezoelectric-based ultrasonic transducers aids in enhancing fibrinolysis [17], tissue penetration [17, 18], clot dissolution [18, 19], thrombolysis [19], and wound healing therapy [20]. In this present work, we have selected 40-kHz central frequency-based ultrasonic transmitters for providing modulated ultrasonic waves to the measurement site (fingertip). Further, (i)

easy availability, (ii) medically safe and tolerable [15–20], (iii) less tissue heating [16–20], (iv) high skin tissue penetration [15–20], and (v) low costs of 40-kHz ultrasonic transducers have boosted our motivation for its selection.

### Light wavelength selection

The composition of human blood is complex and constitutes numerous components in it. Further, glucose molecules exhibit very weak signals. The wavelength selection needs careful and well-judged approaches to overcome this particular phenomenon [21].

Kulkarni et al. [2010] and Konig [2004] described that the region extending from 600 to 1100 nm is the “Tissue Optical Window” of the living biological tissues.

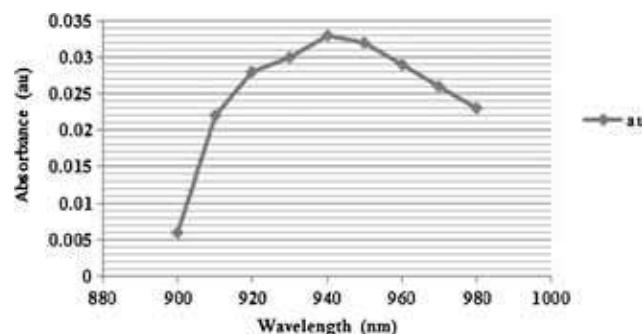
The absorption spectrum of oxyhemoglobin and deoxyhemoglobin is comparatively low within the spectral wavelength band 900 to 1000 nm, respectively. The blood oxygenations vary blood optical absorption properties [22–27].

Khalil [2004] and Khalil [1999] presented that glucose molecules exhibit absorption peaks at the 939-nm (very near to 940 nm) NIR spectral band. So, considering wavelength bands within the “Tissue Optical Window” where water, oxyhemoglobin, deoxyhemoglobin exhibit low absorption profiles and good absorption properties from glucose molecules will be better to avoid any particular noise interferences [26, 27].

Further, for significant wavelength selection, we have performed glucose specificity and sensitivity analysis. The specificity refers here to the particular light wavelength where glucose exhibits maximum absorption peak characteristics. Similarly, the sensitivity refers here to the ability of the particular light wavelength to respond in accordance with the effective variations in the glucose concentration levels.

### Glucose specificity analysis

To determine the maximum wavelength specificity of glucose molecule in distilled water between the 900 and 980 nm wavelength domain, we have prepared a stock solution of 2500 mg dextrose (glucose) per 10 ml of distilled water. From that prepared concentration (*w/v*) stock solution, 2 ml of glucose in distilled water solution has been pipetted out, poured inside the cuvette. The Digital Spectrometer Model 305 of M.S Electronics (India) is used in this present work to measure absorbance (in arbitrary unit=au) of glucose in distilled water solution. Figure 1 depicts the absorption spectra of the glucose in distilled water. Figure 1 reveals that at 940 nm, the glucose in distilled water exhibits maximum absorption peak characteristics within the infrared spectral domain of 900 to 980 nm, respectively. Further, glucose molecule exhibits absorption peak characteristics at 939 nm (very close to 940 nm) due to possible stretch and vibration of the second O-H overtone band in its molecular structure [25, 26].



**Fig. 1** Absorption spectra of glucose in distill water between 900 and 980 nm

### Glucose sensitivity analysis at 940 nm

To determine the 940-nm wavelength degree of sensitivity for respective change in glucose concentration, we have prepared three sample concentration (*w/v*) solutions (dextrose in distilled water) like 2500 mg/10 ml, 5000 mg/10 ml, and 7500 mg/10 ml, respectively.

The Digital Spectrometer Model 305 of M.S Electronics (India) performs the measurement analysis here. Table 1 depicts the output data from those three prepared sample concentration (*w/v*) solutions for glucose sensitivity analysis at a 940-nm wavelength. The output data in absorbance (au) and concentration (parts per million=ppm) presents that the 940-nm wavelength has been sensitive and linearly detects the respective variations of glucose concentration (*w/v*) in the prepared sample solutions.

Hence, various factors like (i) “Tissue Optical Window” range (600 to 1100 nm) [21, 22], (ii) typical peak characteristic of the glucose molecule at 940 nm [21–27], (iii) acceptable specificity cum sensitivity for glucose at 940 nm [21–27], (iv) easy commercial availability [15], and (v) several literatures [28–35] favoring 940 nm for noninvasive blood glucose measurement strongly influenced us for this particular wavelength selection.

### Experimental setup

In this pilot study, we have utilized the modulated ultrasound and infrared light technique-based system. The ultrasonic transmitter and receiver operate at the frequency of  $40.0 \pm 1.0$  kHz. It can resist utmost input voltage of 20 V<sub>rms</sub> and

**Table 1** Glucose sensitivity analysis at 940 nm wavelength

Wavelength (nm)	2 ml from the prepared sample solutions ( <i>w/v</i> )	Absorbance (au)	Concentration (ppm)
940	2500 mg/10 ml	0.033	071
	5000 mg/10 ml	0.045	113
	7500 mg/10 ml	0.067	150

produces a sound pressure output of  $110 \pm 5$  dB at 10 V and 30 cm. In this present work, we have utilized the 940-nm infrared light emitting diode (B5B-940-8) of Roithner LaserTechnik, Vienna, Austria, for irradiating the fingertip of the study subjects. It is a round type with a 5-mm diameter, works with GaAlAs/GaAs technology, and possesses radiated output power ( $P_o$ ) of 32 to 48 mW. Similarly, we have utilized EPD-1300-5.3, InGaAs selective photodiode of Roithner LaserTechnik, Vienna, Austria, for capturing transmitted light from the infrared light (940 nm)-irradiated human finger to measure noninvasive blood glucose levels. This photodiode is perfect for detection of pulsed light with sensitivity starting from 800 to 1750 nm. Figure 2 represents the block diagram of our experimental system utilized in this pilot study.

In this system as depicted in Fig. 2, the carrier wave and the modulating signal unit provides two types of typical sine wave inputs for producing the amplitude-modulated signal waves. This modulated signal serves as an input to the ultrasonic transmitter to provide the amplitude-modulated ultrasonic signal waves to the finger (measurement site) through the finger holder. Here, the ultrasonic receiver unit monitors the characteristics of the generated modulated ultrasonic waves. The modulated ultrasonic wave direction of propagation and the light source point of focus are geometrically perpendicular to the finger-positioning angle. The infrared light focused to the ultrasonic zone of impact produces output as the amplitude-modulated ultrasound light signals. The infrared detector sensitive in the infrared region acquires those specific output signals. The signal amplifier block amplifies the acquired signals. Afterwards, acquired signals were stored in the computer for

further processing through the signal processing toolbox of MATLAB to predict and display the noninvasive blood glucose levels.

## Calibration

The calibration of our experimental system depends upon the peak voltage amplitude (mV) in the FFT domain corresponding to the blood glucose concentration. The calibration factor multiplies with the peak voltage amplitude (mV) in the FFT domain to provide predicted (noninvasive) blood glucose levels in milligram/deciliter and mathematically expressed as:

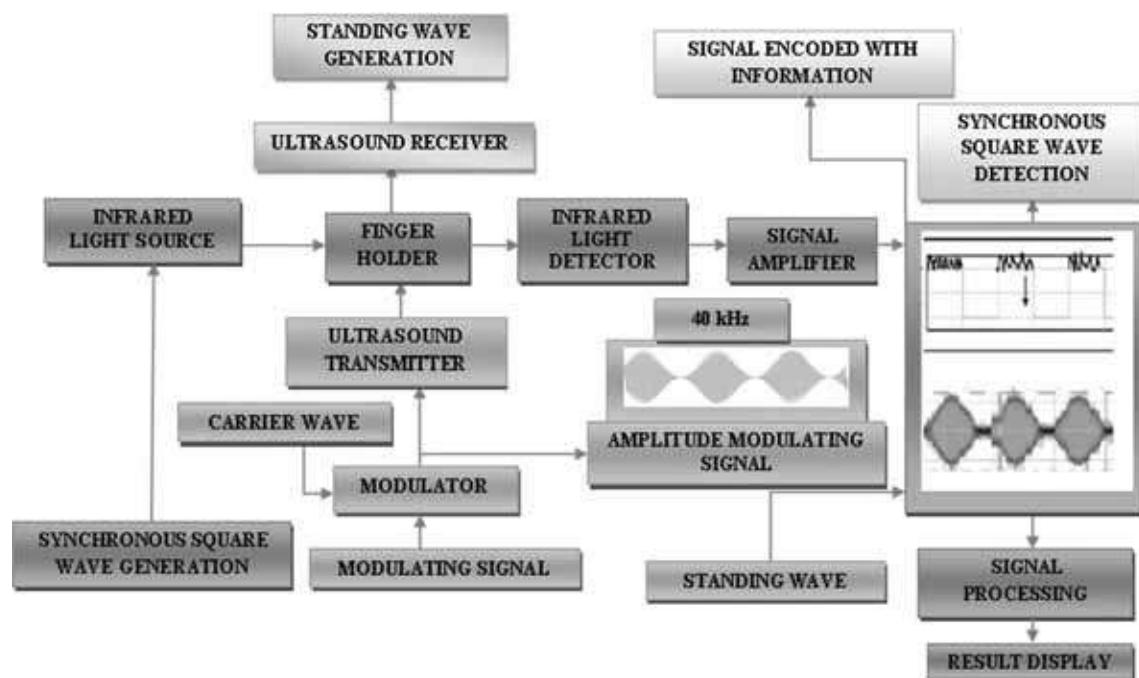
$$V_{\text{peak}} \times \text{CF} = \text{PBGL} \quad (4)$$

where  $V_{\text{peak}}$  stands for peak voltage amplitude (mV) in the FFT domain, CF stands for calibration factor, and PBGL refers to predicted blood glucose level in milligram/deciliter.

## In vitro experiment

In order to establish a noninvasive blood glucose measurement technique, in vitro quasi-finger system (optical tissue phantoms)-based experiments are highly significant. Optical phantoms resemble tissue optical properties, in which various elementary principles and scientific concepts are tested [36–38].

In this present work, our initial approach includes preparing the quasi-finger system to mimic finger absorption and scattering characteristics in the infrared spectral domain. The



**Fig. 2** Noninvasive technique-based blood glucose measuring system

intralipid phantom resembles tissue scattering aspects, while absorption is accounted here by direct addition of the whole blood samples (fasting and postprandial) to it to form the quasi-finger system [36–38].

Mixture of both of these constituents resembles blood tissue medium. The constant level of oxygen in prepared samples is essential to obtain glucose concentration-derived results [36–38].

Herein, *in vitro* (with this prepared quasi-finger system) phantom sample analysis was performed, to explore the glucose sensitivity of our noninvasive technique-based prototype unit.

## Study subjects

In total, 12 adult subjects participated in this pilot study. Two subjects are healthy normal, three subjects had prediabetes, and seven subjects had diabetes. The mean $\pm$ standard deviations of age is 41 $\pm$ 5 years old and mean $\pm$ standard deviations of body mass index is 27.3 $\pm$ 3 kg/m<sup>2</sup>. Overall, ten subjects were male and two subjects were female. The pilot study reported here is in accordance with the standard ethical procedures and performed with the informed consent of all the respective subjects. The pilot study was approved by the Ethical Committee Board of Faculty of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

## Sample preparation

The sample preparation steps include:

- Step 1: Collection of whole blood samples in vacuum-based blood collecting vials, where K<sub>2</sub> EDTA (ethylene diamine tetra acetic acid) is present as an anticoagulant. Addition of phosphate buffer solution (PBS) to maintain the pH levels in the samples
- Step 2: De-oxygenation of the whole blood samples by nitrogen gas bubbling for 45 min to reduce oxygen influence over glucose measurement
- Step 3: One milliliter of the intralipid suspension mixed with 1 ml of whole blood sample to form a quasi-finger system resembling blood tissue optical properties
- Step 4: Placing each prepared phantom samples inside the sample holder of our prototype unit for its respective glucose concentration measurement

The invasive measurements were performed to measure and compare the blood glucose levels with the FFT domain-based peak voltage amplitude (mV).

Table 2 represents the voltage amplitude in the FFT domain during fasting stage and its effective correlation with the invasive blood glucose levels. Similarly, Table 3 represents the voltage amplitude in the FFT domain during postprandial

**Table 2** In vitro glucose sensitivity in phantom samples of fasting stage

Study subject	Invasive fasting blood glucose level (mg/dl)	Peak voltage amplitude (mV) in the FFT domain during fasting stage
Healthy subject 1	87	8.9
Healthy subject 2	89	9.2
Prediabetic subject 3	124	12.9
Prediabetic subject 4	113	11.6
Prediabetic subject 5	119	12.3
Diabetic subject 6	179	18.3
Diabetic subject 7	221	23.8
Diabetic subject 8	168	17.8
Diabetic subject 9	171	16.6
Diabetic subject 10	164	17.2
Diabetic subject 11	201	19.8
Diabetic subject 12	233	22.7

stage and its effective correlation with the invasive blood glucose levels.

In both Tables 2 and 3, the voltage amplitude in the FFT domain changes in correlation with the variation of invasive blood glucose levels.

These correlated changes indicate towards the sensitivity (at 940 nm) of our prototype unit in detecting glucose concentration variations in respective quasi-finger system based *in vitro* samples.

Further, voltage amplitude in the FFT domain increases with increase in blood glucose levels, this phenomenon indicates towards the glucose concentration-induced light clearing

**Table 3** In vitro glucose sensitivity in phantom samples of postprandial stage

Study subject	Invasive postprandial blood glucose level (mg/dl)	Peak voltage amplitude (mV) in the FFT domain during postprandial stage
Healthy subject 1	103	10.5
Healthy subject 2	109	11.3
Prediabetic subject 3	159	16.5
Prediabetic subject 4	164	17.2
Prediabetic subject 5	153	15.8
Diabetic subject 6	261	25.9
Diabetic subject 7	311	31.9
Diabetic subject 8	307	32.2
Diabetic subject 9	272	28.9
Diabetic subject 10	291	30.8
Diabetic subject 11	288	31.5
Diabetic subject 12	301	33.7

effects. As increase in glucose concentration in vitro samples causes reduction of the scattering effects, which minimizes the mismatch of the refractive index acquiescently, increase in light transmission occurs [36–38].

The effective correlation in vitro experiment forms the benchmark as well as foundation for performing in vivo experiments to establish the performance and efficacy of our technique in blood glucose measurement.

## In vivo experiment

### Study subjects

In total, 60 adult subjects participated in this pilot study, out of which, 30 healthy adult subjects participated for OGTT analysis. Next, 30 adult subjects participated for random blood glucose level tests. Eighteen subjects are healthy normal, seven subjects had prediabetes, and five subjects had diabetes. All the prediabetic and diabetic subjects followed their normal routine of meal intake and medications. The mean $\pm$ standard deviations of age is  $40\pm 4$  years old and mean $\pm$ standard deviations of body mass index is  $26.2\pm 2 \text{ kg/m}^2$ . Overall, 44 subjects were male and 16 subjects were female. The pilot study reported here is in accordance with the standard ethical procedures and performed with the informed consent of all the respective subjects. The pilot study was approved by the Ethical Committee Board of Faculty of Medicine, Institute of Medical Sciences, Banaras Hindu University; Varanasi, India.

### Experimental protocol

In this present work, the pilot study consists of two phases (oral glucose tolerance test and random test) to validate the clinical correlation between the invasive technique- and noninvasive technique-based blood glucose levels.

During the first phase, we have performed oral glucose tolerance tests over healthy subjects after their overnight fasting. The duration of each experiment was 2 h and 45 min (10 to 15 min for baseline observation before intake of 75-g glucose solution). The invasive and noninvasive data were recorded every 30 min from the right and left-hand fingers, respectively.

The Accu Chek Active of Roche Diagnostics, Germany [39] and our technique-based system performed reference (invasive) and predicted (noninvasive) blood glucose measurement here, respectively.

Throughout the investigation, the study subjects remain static to reduce motion artifacts and intake of any food or liquids were restricted.

During the second phase, we have performed random blood glucose level analysis (both invasive and noninvasive) over normal, prediabetic, and diabetic subjects, respectively.

## Result and discussion

In this present work, for result analysis, we have performed Clarke error analysis and statistical analysis.

The Clarke error grid analysis critically evaluates the medical importance of the differences between noninvasive (predicted) and invasive (reference) blood glucose measurements.

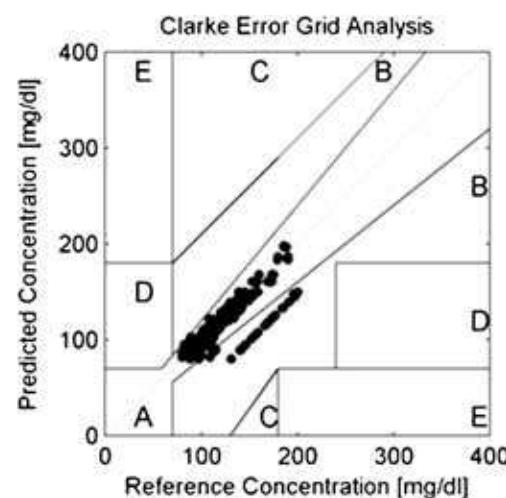
The Clarke Error Grid analysis has been the universal approach for evaluating medical significance of the developing glucose sensor (mostly noninvasive)-based techniques for blood glucose determination.

The Clarke Error Grid consist of five different zones in the XY-Cartesian graph with the following interpretations: Zone A: medically accurate, Zone B: medically significant and tolerable, and Zone C to E: medically insignificant and dangerous. Further, the diagonal line where  $X=Y$ , expresses the ideal measurements. The data points below and above the diagonal line represent the overestimation and underestimation of the real blood glucose values, respectively. When any data pair points fall over the borderline of any zones, the nearness of its (X, Y) coordinate towards either zone determines its zone of occupancy [40–46].

Figure 3 depicts the Clarke Error Grid analysis of the oral glucose tolerance test (180 data pairs)-based BGL data measured by our noninvasive system in comparison to the BGL data measured by invasive device (Accu Chek Active of Roche Diagnostics, Germany).

The Clarke Error Grid analysis presents as follows: A zone=78.33 % and B zone=21.66 %, and none of the values in C to E zones. Hence, oral glucose tolerance test yields that all the noninvasive blood glucose readings occupy A and B zones which are medically important and acceptable.

Table 4 represents invasive and noninvasive blood glucose levels as measured during random blood glucose tests in over 30 study subjects. Figure 4 represents the Clarke Error Grid



**Fig. 3** Clarke Error Analysis of blood glucose data as measured during oral glucose tolerance tests

analysis of the random blood glucose measurement. The Clarke Error Grid Analysis depicts that all the measurements occupy A and B zones (Zone A=83.33 %, Zone B=16.66 %).

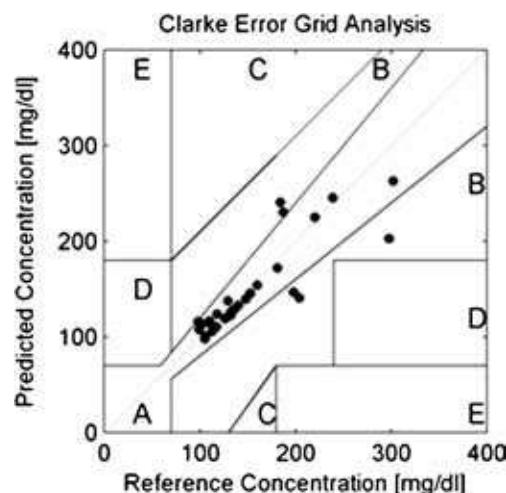
Hence, the random blood glucose measurement represents that all the noninvasive readings are medically significant and acceptable.

Now, the statistical analysis applied here evaluates the blood glucose levels measuring accuracy of our noninvasive technique-based prototype unit during oral glucose tolerance test and random blood glucose measurements.

Table 5 represents the mathematical expressions of the accuracy measure parameters utilized in this present work over the  $N$  number of total samples. All these accuracy measure evaluates the correctness of predicted noninvasive blood glucose levels with respect to the reference invasive blood glucose levels.

**Table 4** Invasive and noninvasive blood glucose levels measured during random blood glucose tests

Subjects	Reference (invasive) BGL (mg/dl)	Predicted (noninvasive) BGL (mg/dl)
01	105	98
02	153	146
03	98	116
04	130	122
05	302	263
06	118	124
07	181	172
08	126	119
09	148	140
10	133	127
11	136	129
12	184	241
13	110	117
14	113	106
15	198	147
16	132	123
17	140	133
18	220	225
19	110	115
20	204	141
21	106	101
22	130	122
23	239	246
24	117	111
25	298	203
26	160	154
27	103	114
28	129	138
29	187	231
30	99	108



**Fig. 4** Clarke Error Analysis of blood glucose data as measured during random blood glucose measurements

Table 6 depicts the statistical analysis-based summary of invasive vs. noninvasive accuracy measures obtained during the pilot study over study subjects. The performance metrics-based errors MAE, MSE, and RMSE values range from 15.92 mg/dl to 17.76 mg/dl, 564.78 mg<sup>2</sup>/dl<sup>2</sup> to 795.76 mg<sup>2</sup>/dl<sup>2</sup>, and 23.76 mg/dl to 28.20 mg/dl, respectively. The performance metrics-based relative errors MARE, MSRE, and RMSRE values range from 0.11 mg/dl/min to 0.10 mg/dl/min, 0.02 mg<sup>2</sup>/dl<sup>2</sup>/min<sup>2</sup> to 0.01 mg<sup>2</sup>/dl<sup>2</sup>/min<sup>2</sup>, and 0.15 mg/dl/min to 0.13 mg/dl/min, respectively. Similarly, the performance metrics-based percentage errors MAPE, MSPE, and RMSPE values range from 11.09 % to 10.10 %, 232.48 % squared to 181.40 % squared, and 15.24 % to 13.46 %, respectively. Further, the correlation coefficient ( $R$  value) value ranges from 0.76 to 0.85 respectively.

Table 6 depicts that our root mean square error (RMSE) for oral glucose tolerance test and random blood glucose measurement was 23.76 mg/dl and 28.20 mg/dl, respectively. These values are significantly comparable with other noninvasive technique-based ranges that extend from 25 to 46 mg/dl [46, 51, 52].

Further, Table 6 depicts the  $R$  value (Pearson correlation coefficient) for oral glucose tolerance test and random blood glucose measurement was 0.76 and 0.85, respectively. These values are important and comparable with the  $R$  value (correlation coefficient) of various other in vivo noninvasive techniques, which extends from 0.49 to 0.95 [51, 52], respectively.

From the perspective of the Clarke and Error Grid analysis, all the noninvasive readings occupy medically significant zones (A and B) and none of the measurements occupy medically insignificant zones (C–E).

Hence, all the overlaid findings including in vitro analysis, Clarke error grid analysis, and performance metrics-based

**Table 5** Accuracy measure parameters

Accuracy measure	Mathematical expression	Symbol notations	References
MAE (mean absolute error)	$\frac{\sum_{i=1}^N  y_i - \hat{y}_i }{N}$	Where $y_i$ is the reference blood glucose level (RBGL), $\hat{y}_i$ is the predicted blood glucose level (PBGL), and $N$ represents the total number of samples	[47–49]
MSE (mean squared error)	$\frac{\sum_{i=1}^N (y_i - \hat{y}_i)^2}{N}$		
RMSE (root mean squared error)	$\sqrt{\frac{\sum_{i=1}^N (y_i - \hat{y}_i)^2}{N}}$		
MARE (mean absolute relative error)	$\frac{\sum_{i=1}^N \left  \frac{y_i - \hat{y}_i}{y_i} \right }{N}$		
MSRE (mean squared relative error)	$\frac{\sum_{i=1}^N \left( \frac{y_i - \hat{y}_i}{y_i} \right)^2}{N}$		
RMSRE (root mean squared relative error)	$\sqrt{\frac{\sum_{i=1}^N \left( \frac{y_i - \hat{y}_i}{y_i} \right)^2}{N}}$		
MAPE (mean absolute percentage error)	$\frac{\sum_{i=1}^N \left  \frac{y_i - \hat{y}_i}{y_i} \right  \times 100}{N}$		
MSPE (mean squared percentage error)	$\frac{\sum_{i=1}^N \left[ \left( \frac{y_i - \hat{y}_i}{y_i} \right) \times 100 \right]^2}{N}$		
RMSPE (root mean squared percentage error)	$\sqrt{\frac{\sum_{i=1}^N \left[ \left( \frac{y_i - \hat{y}_i}{y_i} \right) \times 100 \right]^2}{N}}$		
Pearson correlation coefficient ( $R$ value)	$\frac{N(\sum xy) - (\sum x)(\sum y)}{\sqrt{[N\sum x^2 - (\sum x)^2][N\sum y^2 - (\sum y)^2]}}$	Where $x$ represents RBGL value, $y$ represents PBGL value, and $N$ represents total number of samples.	[50]

**Table 6** The accuracy measurement summary of OGTT and random blood glucose measurement

Statistic function (unit)	Accuracy measurement	
	Oral glucose tolerance test	Random blood glucose measurement
MAE (mg/dl)	15.92	17.76
MSE (mg <sup>2</sup> /dl <sup>2</sup> )	564.78	795.76
RMSE (mg/dl)	23.76	28.20
MARE (mg/dl/min)	0.11	0.10
MSRE (mg <sup>2</sup> /dl <sup>2</sup> /min <sup>2</sup> )	0.02	0.01
RMSRE (mg/dl/min)	0.15	0.13
MAPE (%)	11.09	10.10
MSPE (% squared)	232.48	181.40
RMSPE (%)	15.24	13.46
$R$ value	0.76	0.85

accuracy analysis depict the strong promising aspect of our noninvasive technique for blood glucose measurement in the human subjects.

However, certain error-induced bio-signals were observed due to multiple superfluous causes such as finger placement, finger shape and size, motion artifacts, time and machine drift issues, melanin-induced skin pigmentations, variation in multiple physiological parameters (blood pressure, heart rate, skin sweating, body temperature), and environmental changes, which changes blood tissue optical characteristics and induce variations in the signal acquisition processes. In future works, acquiring preventive measures is essential to reduce the abovementioned interferences.

## Conclusion

We have represented the indigenously designed modulated ultrasound and infrared technique-based system for

noninvasive blood glucose measurement on human subjects. Both the in vitro and in vivo results showed good correlation in glucose measurements.

Further, the result of the Clarke error grid analysis and statistical evaluations values validates that our noninvasive system is potentially capable in performing noninvasive blood glucose measurement over human subjects.

Our noninvasive system was medically safe, easy, and acceptable, as reflected by the overall study subject's well-tolerated compliances.

Therefore, a new technique for noninvasive blood glucose measurement using modulated ultrasound and infrared technique is developed and the observation validates the supposition of the new concept. Positively, our technique will show its functional usage in the future for noninvasive estimations of blood glucose levels in human subjects.

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**Authors' contribution** Md. Koushik Chowdhury, the Doctoral student, wrote the manuscript and is the corresponding author of the manuscript. Md. Koushik Chowdhury and Anuj Srivastava performed experimentations and data collection during the studies. Dr. Neeraj Sharma (associate professor) and Dr. Shiru Sharma (assistant professor) helped in overall supervision for the experimentations, final editing of the manuscript, and getting the necessary formal applications required for the experimental purposes.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Research involving human participants and/or animals-ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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ORIGINAL ARTICLE

## Role of *PPARG* (Pro12Ala) in Malaysian type 2 diabetes mellitus patients

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**Abstract** Diabetes is one of the major lifestyle disorders in the world. Asian countries, including Malaysia, contribute with more than 60 % of the world's diabetic population. The single nucleotide polymorphisms (SNPs) of the peroxisome proliferator-activated receptor gamma (*PPARG*) have been identified as one of the key regulators of glucose and lipid metabolism that controls the protein synthesis in multiple metabolic, biochemical, and molecular pathways. The aim of this study was to investigate the possible role of *PPARG* (Pro12Ala) gene polymorphism as a genetic risk factor for type 2 diabetes mellitus (T2DM) patients in Malaysian population. A total of 241 subjects between the age of 35 and 85 years were recruited in this study. Out of the total 241 subjects, 120 were T2DM patients and 121 were healthy

individuals. SNP of *PPARG* (Pro12Ala) was determined by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs). The frequencies of wild homozygote (WH), heterozygote (H), and mutant homozygote (MH) among the T2DM patients were ( $n=73$ ) 60.8 %, ( $n=39$ ) 32.5 %, and ( $n=3$ ) 2.5 % compared to ( $n=57$ ) 47 %, ( $n=46$ ) 38 %, and ( $n=16$ ) 13.2 % among the healthy subjects. The mean of Hba1C (%) among normal and diabetic patients with genotypes were different ( $5.36 \pm 0.54$  vs  $7.58 \pm 1.76$ ,  $p < 0.005$ ). SNP of *PPARG* (Pro12Ala) gene could be a genetic risk factor for insulin resistance and T2DM among Malaysian population.

**Keywords** Single nucleotide polymorphism · Peroxisome proliferator-activated receptor gamma · Type 2 diabetes mellitus · Genotype · Insulin resistant

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### Introduction

Type 2 diabetes mellitus (T2DM) has become a common global health problem which has affected nearly 350 million people worldwide. The number of people with T2DM is increasing at an alarming rate [1–3]. T2DM is a heterogeneous disorder which is characterized by an impaired insulin secretion and insulin resistance, and also both genetic and environmental factors contribute to the disease risk [4–6].

Insulin resistance (IR) and insulin secretion (IS) are two main characteristic of T2DM and play a vital role in the pathogenesis of the disease. Such IR and IS defects have been shown through several alterations in carbohydrates, lipids, and protein metabolism [7–9]. Diabetes is also accompanied by over production of free radicals, which consequently contributes to the generation of reactive oxygen species (ROS). Besides ROS production, a growing body of evidence

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suggests that there is a link between diabetes and mitochondrial functioning [10, 11]. Diabetes is typically accompanied by an increased production of free radicals and/or by impaired antioxidant defense capabilities, indicating a central contribution for ROS in the onset, progression, and pathological consequences of diabetes. Besides oxidative stress, a growing body of evidence has demonstrated a link between various disturbances in mitochondrial functioning and type 2 diabetes.

In addition to IR, IS, and environmental factors, various genes also play a significant role in the progression of T2DM. Recent, genome-wide association studies (GWAS) have successfully identified and replicated nearly 75 novel [12] and candidate genes as T2DM susceptibility loci, including genes that are affecting insulin secretion and insulin sensitivity [13]. This is predominantly found in European African and South Asian populations [12]. Due to the discrepancy of ethnicity in allelic and linkage disequilibrium (LD) architecture, replication studies of genes in other population show a casual variant in ethnic-specific populations [14].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are a subfamily of the nuclear receptor gene family. Three types of PPAR ( $\alpha$ ,  $\gamma$ , and the ubiquitously expressed  $\beta/\delta$ ) have been described in mammals [15]. Previous studies have been demonstrated that peroxisome proliferator-activated receptor gamma (*PPARG*) is a nuclear receptor that regulates adipocyte differentiation and plays an important role in controlling lipid and glucose metabolism. It is also implicated in various metabolic diseases such as hyperlipidemia, diabetes mellitus, and coronary artery disease (CAD) [16]. *PPARG* also plays a significant role as transcription factor, mediating regulatory mechanisms in metabolism, inflammation, endothelial function, cancer, and bone morphogenesis [17].

Ten protein-coding splice isoforms and two nonsense mediated decay splice isoforms ([www.ensembl.org](http://www.ensembl.org)) of *PPARG* are *PPARG1* and *PPARG2* which are expressed in adipose, heart, muscle, and liver tissues. Moreover, the splice variant ENST00000397026 (*PPARG-005*) has a threonine at position 12 of the coded protein. In these tissues *PPARG1* is much more abundant than *PPARG2* [18]. Both isoforms possess ligand-dependent and ligand-independent domains. However, *PPARG2* has an additional 28 amino acids in the NH<sub>2</sub>-terminus that renders its ligand-independent activity 5–10 times higher than that of *PPARG1*. This ligand-independent activation requires insulin stimulation [19]. It has also been shown that natural *PPARG* ligands, such as prostaglandins [20], fatty acids [21], or synthetic ligands such as thiazolidinediones (TZDs), bind to *PPARG* and lead to ligand-dependent activation. Activation of the *PPARG* by TZD, a group of antidiabetic drugs, can lead to increased insulin sensitivity and improved glucose tolerance in patients with T2DM [22]. Therefore, this drug is currently used as an effective treatment for diabetes.

Several studies suggest that *PPARG* activation causes insulin sensitization. First, the in vitro binding affinity of TZD ligands to *PPARG* correlates well with their in vivo potency as insulin sensitizers [23, 24]. Second, this relationship with *PPARG* activation is shared by other ligands that are not structurally related to TZDs [23]. Third, retinoid X receptor (RXR) ligands, which can activate the *PPARG* RXR heterodimer, also have insulin sensitizing effects in rodents [25]. Fourth, humans with a polymorphism in the gene encoding *PPARG* that marginally alters *PPARG* activity (Pro12Ala) are more sensitive to insulin [26]. Fifth, mice that possess a mutation that increases *PPARG* activity by preventing its phosphorylation at Ser112 of *PPARG2* are protected from obesity-associated insulin resistance [27]. Surprisingly, mice that lack one allele of the *PPARG* gene are more sensitive to insulin than their wild-type littermates [28, 29]. The reasons for this are not clear, but this effect might be caused by alleviation of gene repression by the reduced level of *PPARG* in the absence of saturating concentrations of ligand. In any case, an optimal level of *PPARG* activity is crucial for its beneficial effects as an insulin-sensitizing transcriptional regulator [30].

The single nucleotide polymorphisms (SNPs) are defined as genetic variation in a DNA sequence that occurs when a single nucleotide in a genome is altered. SNP of the *PPARG* have been identified as key regulators of glucose and lipid metabolism that control protein synthesis in multiple metabolic, biochemical, and molecular pathways. Moreover *PPARG* is also involved in adipogenesis and in regulation of adipocyte gene expression and glucose metabolism [31]. Recently, several GWAS in Caucasians [32–34] have confirmed that the most widely known association between genetic variation and population risk in diabetes was *PPARG* (Pro12Ala). Several studies on gene–gene interaction, metabolic pathways, and diabetic and non-diabetic subphenotypes also provided some strong information regarding the potential mechanism by which the Pro12Ala polymorphism reduces the risk for T2DM [35]. Therefore, we sought to investigate the possible role of *PPARG* (Pro12Ala) gene polymorphism as a genetic risk factor for T2DM patients in Malaysian population.

## Research design and methods

### Study subjects

This study was carried out on 241 patients who visited the diabetic clinic at University of Malaya Medical Centre (UMMC) between 2013 and 2014 and who agreed to participate upon our explanation of the purpose and procedures of the study.

All diabetic patients were at age >30 and all other chronic disease was excluded for this study. This study was approved

by an internal ethics committee (UMMC). A total of 121 healthy subjects were recruited in this study as controls.

## Criteria

Inclusion: > 30 years, T2DM, early stage of diabetes

Exclusion: chronic or diabetic complications

## Anthropometry

The height, weight, systolic, and diastolic blood pressures were measured for each patient by a registered nurse and recorded in medical folder. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Hypertension was defined as blood pressure >140/90 mm Hg or current use of antihypertensive medications. Patients who had smoked regularly in the previous years were considered active smokers.

## Blood collection and plasma separation

Venous blood samples were obtained from the subjects after 8 h of overnight fasting in vacutainers with and without appropriate anticoagulants. Immediately, the plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min at 4 °C. Later, plasma was stored at –20 °C for further biochemical analysis.

## Biochemical analysis

Biochemical parameters related to T2DM were estimated for both cases and control subjects. Measurement of serum levels of total cholesterol (TC), triglycerides (TG), HbA1c, fasting blood glucose (FBG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), C-reactive protein (CRP), and creatinine were measured spectrophotometrically using an automated clinical chemistry analyzer, Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany) [36].

## DNA isolation

Genomic DNA was extracted from whole blood using standard method described by Qiagen company [37].

## Genotyping

### *PPARG (Pro12Ala polymorphism)*

The PCR was carried out in a final volume of 20 µl containing 0.2 µl of genomic DNA, 10 µl of 1×PCR Master Mix (Gendiex, USA), 2 µl of forward and reverse primers, and 7.8 µl of dH<sub>2</sub>O. The oligonucleotide sequences (Forward primer:

5'-GCCAATTCAAGCCCAGTC-3' and Reverse primer: 5'-GATATGTTGCAGACAGTGTATCAGTGAAGGAATCGCTTCCG-3') were used to amplify the DNA. After an initial denaturation of 3 min at 94 °C, the samples were subjected to 35 cycles at 94 °C for 30 s, at 64 °C (annealing) for 30 s, and 72 °C for 1 min, with a final extension of 500 s at 72 °C in a mega cycler (Edvotek 542, China). The PCR products were digested by enzyme restriction *Bsh 1236i* for *PPARG* (Thermo Scientific) and were separated by 1.5 % agarose gel electrophoresis at 100 °C, 80–90 V, and 150 mA for 1 h. The allele encoding wild homozygote appeared as a single fragment of 270 bp, mutant homozygote appeared as a double fragment of 227 and 43 bp, and heterozygote appeared as triple fragment of 270, 227, and 43 bp.

## Statistical analysis

SPSS 20.0 software was used to analyze the data. All the continuous variables are presented as mean±standard deviation (SD). Student's *t* test was used to calculate the *p* values. Genotype and allele frequency were analyzed using the chi-square test, Hardy–Weinberg equilibrium, and odd ratio (OR) with *p* values. Biochemical tests were performed and the findings were tabulated.

## Results

### Anthropometric results

The descriptive data and comparison of anthropometric parameters of diabetic patients versus controls are shown in Table 1. The study was carried out in a total of 241 subjects which grouped 120 subjects as T2DM (male *n*=60 and female *n*=60) and 121 subjects (male *n*=60 and female *n*=60) as control. Age of the subjects was respectively 55.2±8.7 years for T2DM with a mean BMI 28.1±5.2 kg/m<sup>2</sup> and 7.5±13.0 years for control with a mean BMI 26.8±4.2.

### Biochemical and clinical findings

The descriptive data and comparison of biochemical parameters for diabetic patients versus controls are presented in Table 2. According to our results, diabetic patients had markedly higher levels of total cholesterol (*p*<0.005), LDL-C (*p*<0.005), HbA1c (*p*<0.005), and FBG (*p*<0.005) compared with those of controls. Differences were also observed for TG (*p*=0.857), HDL-C (*p*=0.891), CRP (*p*=0.281), and creatinine (*p*=0.432). TG values, HDL-C levels, creatinine values, and CRP values were not significantly different between the two groups.

**Table 1** Comparison of anthropometric parameters between diabetic patients and control

Parameters	DM (n = 120)	Control (n = 121)	p Value
Age	55.2 ± 8.7	47.5 ± 13.0	<0.005 <sup>a</sup>
Weight (kg)	70.5 ± 13.7	68.5 ± 10.7	0.226
Height (cm)	158.5 ± 6.7	159.9 ± 8.1	0.152
BMI (kg/m <sup>2</sup> )	28.1 ± 5.2	26.8 ± 4.2	0.045
Systolic_BP (mm Hg)	132.9 ± 16.1	128.9 ± 12.8	0.036
Diastolic_BP (mm Hg)	76.0 ± 10.0	76.8 ± 10.7	0.591
Smoking (%)	10.8	10.7	
Alcohol (%)	10.8	10.7	
Hypertension (%)	65	2.48	

<sup>a</sup>The mean difference is significant at the  $p < 0.005$ . Values expressed as mean ± SD are taken at one point of time during treatment and will not indicate a lifelong trend of the concentrations in the given patients

These were calculated using fasting plasma glucose (mmol/l) and fasting plasma insulin (mU/l) levels.

### Detection of *PPARG* (Pro12Ala)

The genotype distribution of *PPARG* (Pro12Ala) was strongly under the Hardy–Weinberg equilibrium ( $\chi^2 = 0.77$  and 2.78 for T2DM and controls, respectively). The observed genotype and allele frequencies for *PPARG* (Pro12Ala) polymorphism are shown in Table 3. Moreover, T2DM subjects had the higher frequency of wild-type genotype (ProPro) ( $p = 0.021$ ) than control. Besides that, the mutant genotype AlaAla was present at a lower frequency ( $p = 0.056$ ) in the T2DM group as compared to the control group (8.0 vs 18.0 %). An OR of 2.88 in the diabetic patients group indicates association of the

**Table 2** Comparison of biochemical and clinical findings between diabetic patients and control

Parameters	DM (n = 120)	Control (n = 121)	p Value
TG (mmol/L)	1.72 ± 0.85	1.70 ± 0.87	0.857
TC (mmol/L)	4.82 ± 1.14	5.39 ± 1.19	<0.005 <sup>a</sup>
HDL-C (mmol/L)	1.44 ± 0.84	1.45 ± 0.72	0.891
LDL-C (mmol/L)	2.72 ± 0.98	3.29 ± 1.04	<0.005 <sup>a</sup>
HbA1c NGSP (%)	7.58 ± 1.76	5.36 ± 0.54	<0.005 <sup>a</sup>
FBG (mmol/L)	9.70 ± 3.17	5.67 ± 0.97	<0.005 <sup>a</sup>
CRP (mg/dL)	0.59 ± 0.66	0.50 ± 0.74	0.281
Creatinine (μmol/L)	84.05 ± 42.53	74.90 ± 24.65	0.432

TG triglycerides, TC total cholesterol, HDL-C high density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, HbA1c glycated hemoglobin, FBG fasting blood glucose, CRP c reactive protein

<sup>a</sup>The mean difference is significant at the  $p < 0.005$ . Values expressed as mean ± SD are taken at one point of time during treatment and will not indicate a lifelong trend of the concentrations in the given patients.

common genotype (ProPro) with T2DM. The OR of a person with the major Pro allele being T2DM was 1.72 times higher as compared to Ala allele. The genotype and allele distribution of T2DM and control groups suggest a significant association of *PPARG* (Pro12Ala) genotype (ProPro) as shown in Table 3.

### Discussion

T2DM is a well-known multifactorial disease which is strongly associated with both genetic and environment factors. *PPARG* (Pro12Ala) gene polymorphism is one of the most common variants which are mostly expressed in adipose tissue. The major role of *PPARG* in glucose and lipid are well documented. Furthermore, 80 % of people had the Pro12Ala gene polymorphism while more than 25 % of them are presumed to be at risk for diabetes. [26]. Previous studies have reported that Pro12Ala polymorphism of the *PPARG* gene is implicated in T2DM in various ethnic populations of the world [14, 38–40]. This is the first study carried out in Malaysian population to determine the association between Pro12Ala polymorphism of the *PPARG* in T2DM patients. This study was designed to increase the understanding of the association between Pro12Ala polymorphism of *PPARG* gene with T2DM and its possible role in the progression of the disease using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP). The finding of the present study would improve the understanding of the pathophysiology of T2DM. In future, our results may help to provide new opportunities for treatment, diagnosis, and monitoring of T2DM in Malaysian population.

Our results demonstrate that the major Pro allele present with a significantly higher frequency in diabetic subjects as compared to control group ( $p < 0.005$ ; OR = 1.72, 95 % CI 1.15–2.58), while the minor Ala allele appeared with significantly lower frequency in diabetic subjects ( $p < 0.005$ ; OR = 1.00). This study suggests, as previously mentioned by Arvind et al. (2013), that minor Ala allele may play a protective role in the pathophysiology of T2DM [17]. Our results support several studies conducted in different populations of the world [17, 39, 41–43]. Consistent with our result, it has been reported that the protective effect of the less common allele Ala occurred at a lower frequency in T2DM subjects than in controls in central Indian population [17] but a little lower frequency in Punjabi Sikh [44] and west Bengal populations [41]. The similar results have been reported from studies in Japanese [42] and Finnish populations [43]. In addition, our data support the protective role of Ala allele in T2DM patients in the Malaysian population.

A meta-analysis study by Altshuler et al. (2000) [26] has reported that the presence of Ala allele plays ~20 % of reduction in the onset of T2DM. If Ala allele of Pro12Ala SNP had a protective effect on diabetes risk on various ethnic groups, one

**Table 3** Genotypic distribution of the *PPARG* gene

<i>PPARG</i> genotype	T2DM ( <i>n</i> = 120), n (%)	Controls ( <i>n</i> = 121), n (%)	<i>p</i> Value	Odds ratio (95 % CI)
ProPro (WH)	73.0 (60.83)	57.0 (47.11)	<0.005	2.88(1.17–7.1)
ProAla (H)	39.0 (32.50)	46.0 (38.02)	0.176	1.91(0.75–4.86)
AlaAla (MH)	8.0 (6.67)	18.0 (14.88)	0.056	1.00
Alleles				
Pro	185 (77.08)	160 (66.12)	<0.005	1.72(1.15–2.58)
Ala	55 (22.92)	82 (33.88)	<0.005	1.00

Adjusted for age, sex, BMI, hypertension, and diabetes. For the other abbreviations used, see Tables 1 and 2  
*CI* confidence interval, *OR* odds ratio.

would expect lower Ala allele frequency in populations characterized by a high prevalence of diabetes, such as the South Asians. Besides that, our study also support the report of Venkatesan et al., who found that Ala allele frequency in South Asians and in Caucasians are ~10 and ~11 %, respectively [45]. The ethnic differences in relation to the protective role of Ala allele for T2DM risk may in fact have both genetic and environmental origins.

Another study with large amount of Chinese population (3146 samples) revealed a significant protective association of Ala allele ( $OR=0.67$ , 95 % CI 0.52–0.86), while an OR of 0.65 which is slightly lower for the less common Ala allele was identified among an East Asian population [39, 40]. Besides that, another meta-analysis of eight studies on Japanese and Caucasian populations reported an identical OR of 0.81 for the same Ala allele [46, 47].

The Ala allele at codon 12 of *PPARG* gene plays protective role in T2DM patients and consistently reproduced in multiple independent studies conducted in different populations [16, 48, 49]. Majority of the studies have found that the carriers of Ala allele play minimum role in the progression and development of T2DM and insulin resistance [48–51]; however, contrary to our study, few studies are at odd to this association [52, 53]. Several studies include a study of South Indian populations, and Canadian Oji-Cree populations have suggested that the Ala allele is associated with a higher risk of T2DM and indicated that the allele does not have any protective effects [54, 55].

Moreover, studies on animal models such as knockout mice have also supported the results in human subjects [29]. The association between Pro12Ala polymorphism and T2DM is well documented in Japanese, Scandinavian, Polish, Finish, Italian, and other populations [48, 56–61]; however, no study has been conducted to investigate this association in Malaysian population. Besides that, these polymorphism have been also tested and the studies were published on microvascular complication on T2DM. These studies show that the alanine variant was protective against diabetic nephropathy in German [62] and Brazilian [63] populations. However, the above

studies have proved that multiple ethnic groups, different origin, and ethnicity are those factors that modulate the effects of this particular polymorphism in predicting risk for T2DM.

In contrast to the current study, [59, 64] few studies failed to found relationship between the Pro12Ala polymorphism of *PPARG* gene and T2DM. Population and ethnic difference may affect the role of Pro allele as a risk factor for T2DM by many theories such as the genetic background because of the gene–gene interaction or nutrition habits as some foods are considered *PPARG* ligands like oleic acid as reported by Soriquer et al. (2006) [65].

Our results coincide with Soriquer et al. (2006) [65] who had reported that Pro12Ala polymorphism of *PPARG* gene (Pro allele) is associated with insulin resistance especially in population with a high intake of oleic acid and suggested an interaction between this gene polymorphism and dietary intake especially monounsaturated fatty acids which are considered as *PPARG* ligands.

It explains that alanine helps to form  $\alpha$ -helix while proline prevents it. Thus, change in this amino acid will affect the structure and consequently the function of the protein [66]. This may improve the insulin sensitivity, and it is known that decreased insulin sensitivity plays an important role in the pathogenesis of T2DM [67].

We found that the Ala allele of the Pro12Ala polymorphism is associated with a significantly lower risk of T2DM in Malaysian population. The frequencies of the Ala allele in patients with and without diabetes were 22.92 and 33.88 %, respectively, in Malaysian population. The samples with low frequency of Ala allele as compared to our study was in Caucasian (9 %) [35], Korean (9.6 %) [68], African (2.3 %) [69], Iranian (9.4 %) [70], West Bengal (9 %) [41], and Punjab Sikh(10 %) [44] populations.

## Conclusion

In conclusion, our present findings suggest that the inclusion of Ala allele of the common Pro12Ala SNP in the isoform

*PPARG* is associated with a reduce risk or also known to be protection for T2DM in Malaysian populations.

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**Compliance with ethical standards** All procedures performed in this study involving human participants were in accordance with the ethical standards of the University of Malaya.

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ORIGINAL ARTICLE

# A study of paraoxonase1 (PON1) activities, HDL cholesterol and its association with vascular complication in type 2 diabetes mellitus

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**Abstract** The aim of this study is to find out the clinical relevance of estimating serum paraoxonase1 (PON1) arylesterase and PON1 lactonase activity in type 2 diabetes mellitus patients in relation to the development of vascular complications. We have investigated the fasting blood glucose level, HDL cholesterol levels, PON1 arylesterase and PON1 lactonase activities in 80 type 2 diabetes mellitus (DM) patients (DM without complication  $n=40$ , DM with vascular complication  $n=40$ ) and compared with 40 healthy age- and sex-matched controls. PON1 arylesterase (ARE) and lactonase (LACT) activities in DM patients with complications ( $ARE = 60.615 \pm 15.510$  KU/L,  $LACT = 18.056 \pm 4.215$  U/L) are decreased significantly than in DM without complications ( $ARE = 93.507 \pm 21.813$  KU/L,  $LACT = 32.387 \pm 8.918$  U/L) which are also decreased significantly as compared to controls ( $ARE = 159.94 \pm 45.87$  KU/L,  $LACT = 50.625 \pm 6.973$  U/L). Logistic regression analysis is applied for assessing predictive utility for diabetic complications demonstrated a significant contribution of PON1 lactonase (Naglekerke's  $R^2 = 0.625$ , AUC = 0.907) and arylesterase (Naglekerke's  $R^2 = 0.427$ , AUC = 0.853) activities. Decreased PON1 lactonase and arylesterase activities may be considered as an additional risk factor for the development of vascular complications in type 2 DM.

**Keywords** Diabetes mellitus · Arylesterase · Lactonase

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## Abbreviations

PON1	Paraoxonase1
PON2	Paraoxonase2
PON3	Paraoxonase3
HDL	High Density Lipoprotein
HDLc	High Density Lipoprotein cholesterol
LDL	Low Density Lipoprotein
DM	Diabetes Mellitus
ARE	Arylesterase
LACT	Lactonase
AUC	Area under curve
CETP	Cholesterol ester transport protein
LCAT	Lecithin Cholesterol acyl transporter
CVA	Cerebrovascular accidents
PVD	Peripheral vascular diseases
ECG	Electrocardiogram
CT	Computed tomography

## Introduction

Diabetes mellitus is previously considered as a disease of minor significance to world health, but now, it is taking place as one of the main threats to human health in the twenty-first century [1]. It is the most common non-communicable disease worldwide and the fourth or fifth leading cause of death in developed countries [2]. The prevalence of diabetes is currently estimated to be about 6.4 % worldwide, and there has been a dramatic increase in the diagnosis of type 2 diabetes since the past two decades [3]. Developing countries such as India had the maximum increases in the last few years. The current prevalence of type 2 diabetes is 2.4 % in the rural population and 11.6 % in the urban population of India. It has been estimated that by the year 2025, India will have the largest number of diabetic subjects in the world [4].

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels [5]. As the disease progresses, tissue or vascular damage ensues leading to severe diabetic complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration [6].

The current study aims to find out the clinical relevance of estimating serum PON1 arylesterase (EC 3.1.1.2) and PON1 lactonase (E.C. 3.1.1.81) activity in type 2 diabetes mellitus patients in relation to the development of vascular complications.

PON1 is a calcium-dependent glycoprotein consisting of 354 amino acid residues with molecular weights of 43–47 kDa. PON1 is synthesized in the liver and then secreted into the plasma where it is mainly bound to high-density lipoproteins containing apolipoprotein A-1 and clusterin (apolipoprotein J) [7, 8].

Paraoxonase1 (PON1) hydrolyzes organophosphate compounds and fatty acid lactones [9, 10]. It is the high-density lipoprotein-associated enzyme having an antioxidant property with arylesterase and lactonase activities [11, 12].

The PON gene family consists of three members, PON1, PON2 and PON3, located adjacent to each other on the long arm of chromosome 7 in human [13]. PON1 and PON3 associate with HDL in the circulation; PON2 protein is undetectable in HDL, LDL or the media of cultured cells by western blot but appears to remain intracellular, associated with membrane fractions [14]. PON1, PON2 and PON3 possess the ability to hydrolyze lactones with the three PON proteins exhibiting overlapping but also distinct substrate specificities [15].

Human serum paraoxonase levels are inversely related to the risk of cardiovascular diseases. Some studies found that PON1 activity is decreased in cardiovascular and diabetes mellitus [16, 17].

PON1 has been shown to diminish LDL oxidation and prevent the pro-inflammatory response elicited by oxidized LDL, the latter probably resulting from the metabolism of lipid peroxides [18]. Moreover, PON1 has also been shown to be critical for preventing the oxidation of HDL, allowing it to maintain its function [19]. It is also observed that PON1, by inhibiting LDL oxidation, prevents the upregulation of monocyte chemotactic protein-1 (MCP-1) secretion, and this may inhibit atherosclerosis in the early stage [20]. PON1 also found to hydrolyze hydrogen peroxide ( $H_2O_2$ ), a major reactive oxygen species produced under oxidative stress which is important in the pathogenesis of atherosclerosis. Thus, PON1 preserves

antiatherogenic functions of HDL and also protects oxidation of LDL [21].

Cardiovascular disease risk in type 2 DM may also be increased by qualitative changes in LDL subfractions with an increased proportion of LDL occurring as small dense LDL [22].

HDL also exhibits various anti-atherogenic, antioxidant, anti-inflammatory and anti-thrombotic properties. HDL is known to attenuate the development of atherosclerosis by a variety of mechanisms, including the removal of excess cholesterol from cells of the vessel wall (reverse cholesterol transport) and limiting LDL oxidation. An essential role in these beneficial functions may be played by enzymes and proteins associated with this lipoprotein such as cholesterol ester transfer protein (CETP), lecithin-cholesterol acyl transferase (LCAT), paraoxonase1 (PON1) and apolipoprotein A-I [23].

## Method

### Study population

Eighty (80) type 2 DM patients who attended the medical outpatient department of SRTR Govt. Medical College Ambajogai were enrolled in our study. The patients were then divided into two groups based on the presence or absence of (micro- and macrovascular) complications. Forty (40) belonged to type 2 DM with complication and forty (40) belonged to type 2 DM without complication group. Forty age- and sex-matched healthy subjects were selected as control.

Diagnosis of diabetes was based on current criteria of the American Diabetes Association 2010. The presence of macrovascular complications like CVA and coronary artery disease was based on ECG, positive coronary angiography and CT brain findings and that of PVD was based on clinical findings and Doppler studies. Presence of microvascular complications was evaluated by assessment of retinopathy and nephropathy. Prediagnosed cases by physicians were included. Retinopathy was diagnosed on the basis of history and fundus examination and that of nephropathy was done on the basis of history and clinical investigations like microalbuminuria.

### Sample collection

With all aseptic precautions, fasting blood samples were collected in fluoride and plain bulb. Blood from plain bulb was centrifuged at 1000 rpm for 15 min for serum separation and samples were analysed immediately.

### *Plasma glucose estimation*

Plasma glucose was estimated by glucose oxidase peroxidase method [24].

### *Serum HDLc is estimation*

Apolipoprotein B containing lipoproteins present in serum were precipitated by phosphotungstate in the presence of divalent cations such as magnesium. The HDL particles remain unaffected in the supernatant and cholesterol from HDLc was then estimated by cholesterol oxidase peroxidase method [25].

### *Serum PON1 arylesterase activity*

The assay mixture contained 4.0 mM/L phenylacetate, 1 mM/L CaCl<sub>2</sub> dissolved in 20 mM/L Tris HCl buffer, pH 8.0 at 25 °C. Reaction was initiated by adding a 5-μL sample in a 3-mL assay mixture. The rate of phenol formation was recorded at 270 nm following 20 s lag time. One unit of arylesterase activity is equal to 1 mM of phenylacetate hydrolysed per min. The activity was expressed as unit per liter, based on the extinction coefficient of phenol of 1310 M<sup>-1</sup> cm<sup>-1</sup> at 270 nm, pH 8.0, and 25 °C. Blank samples containing water were used to correct non-enzymatic hydrolysis [26].

### *Serum PON1 lactonase activity*

Final assay mixture contained 50 mM Tris HCl of pH 8 and 1 mM CaCl<sub>2</sub>. One millimolar DHC was present in the buffered substrate. Reaction was initiated by adding a 10-μL sample to 2 mL of buffered substrate at 25 °C. Rate of hydrolysis was determined at 270 nm [27].

### **Statistical analyses**

Statistical analysis was carried out using standard statistical methods using statistical software. Data was expressed as mean ± standard deviation. Differences between mean were compared by Student's *t* test. Multiple logistic regression analysis was used to evaluate the association between vascular complication in diabetic patients and variables such as PON1 arylesterase activity, PON1 lactonase activity and HDLc. Results were expressed as odds ratios and 95 % CI. *p*<0.05 indicated statistical significance.

## **Results**

The demographic data and biochemical characteristics of the study subjects are summarized in Table 1. There is no significant difference for age and sex between cases and controls. Other parameters—HDLc, PON1 arylesterase and PON1 lactonase—were found to be decreased significantly in both groups, i.e., persons having type 2 DM with complication and persons having type 2 DM without complication as compared to normal healthy subjects and BSL is increased significantly in both groups compared to that of the control group.

The logistic regression analysis taking diabetic complications as a dependent factor is shown in Table 2. Area under the curve (AUC) and Naglekerke's *R*<sup>2</sup> when logistic regression is applied for only PON1 arylesterase (ARE) are 0.898 and 0.593, respectively, and when PON1 lactonase (LACT) was added, it became 0.986 and 0.885, respectively; when HDLc is added to this, it became 0.997 and 0.946, respectively.

### **Discussion**

In the present study, we have observed that there is a decrease in serum PON1 arylesterase and serum PON1 lactonase activity in persons having type 2 DM with complication group and persons having type 2 DM without complication group as compared to control subjects and more so in persons having type 2 DM with complications.

We also observed that when logistic regression analysis was applied taking diabetic complications as a dependent factor suggest that HDL-associated enzymes PON1 arylesterase (ARE), PON1 lactonase (LACT) and the combination with HDLc have an additional diagnostic significance for the prediction of diabetic complication.

Our results are in agreement with M. Flekac et al. and S. Bansal et al. who have shown a decrement in serum PON1 arylesterase activity in diabetic vascular complication compared to that of control subjects [28, 29]. Various studies are done on the PON1 polymorphism and risk of diabetic complications [30, 31], but to the best of our knowledge, there is no study regarding PON1 lactonase activity and risk of development of diabetic complication. There are various studies to describe a mechanism by which the vascular complications in diabetic patients develop. Development of diabetic complications has been hypothesized to be accelerated by generation of free radicals in cells and tissues [30]. In diabetes, oxidative stress is due in part to an increased production of plasma free radical concentrations and a sharp reduction in antioxidant

**Table 1** Clinical characteristic and laboratory data of diabetic patients and healthy subjects

Parameters	Healthy subjects	Type 2 DM without complication	Type 2 DM with complications
Subjects ( <i>n</i> )	40	40	40
Age (years)	57.32 ± 11.23	56.26 ± 10.09	59.15 ± 13.87
Sex (male/female)	22/18	21/19	20/20
Fasting plasma glucose (mg%)	97.900 ± 21.469	168.65 ± 33.500**	217.02 ± 51.51**##
HDLc (mg%)	50.625 ± 6.937	31.525 ± 4.994*	19.925 ± 3.622**#
ARE (U/L)	159.94 ± 45.87	93.507 ± 21.813**	60.615 ± 15.510***#
LACT (U/L)	50.625 ± 6.973	32.387 ± 8.918*	18.056 ± 4.215**#

\**p* < 0.05 with respect to the control group

\*\**p* < 0.001 with respect to the control group

# *p* < 0.05 with respect to the without complication group

## *p* < 0.001 with respect to the without complication group

defences. Among the causes of enhanced free radical production, hyperglycaemia, hyperinsulinaemia and/or insulin resistance play major roles [31]. It may be postulated that oxidative stress represents the common pathway through which hyperglycemia and insulin resistance induce depressed insulin action [32].

The low PON1 activity leads to decreased ability to prevent lipid peroxide formation with consequent acceleration of the oxidative stress and also modify composition, function and concentration of the HDL. Elevated plasma triglyceride-rich lipoproteins may substitute cholesterol esters (CE) in HDL by driving cholesterol ester transfer protein (CETP) with subsequent HDL depletion of CE as a result; both the conformation and function of HDL may be altered [17]. Glycation of HDL or directly of PON1 in HDL as occurs in diabetes may result in detachment of PON1 itself from the HDL and PON1 inactivation [33]. The low enzyme activity is caused rather by glycation of the PON1 protein than by reduced synthesis [34]. PON1 is bound by HDL in a lesser extent in diabetic patients as compared to healthy persons, and its activity is then poorly stabilized [33, 35].

Our results support an idea that protection against lipid oxidation by PON1 may be reduced in diabetic patients because of a lower enzyme activity. We also have investigated whether it is possible to predict the severity of diabetes mellitus through PON1 activity.

G Betanzos-Cabrera et al. in their study on streptozotocin-induced diabetic mice fed with a high-fat diet showed that the supplementation with pomegranate juice significantly increased PON1 activity compared to mice that did not receive the pomegranate juice. Mice supplemented with pomegranate juice showed the lowest body weight and reduced blood glucose but not demonstrating that pomegranate juice has a hypoglycaemic effect. They also indicated that including this fruit juice in the diet has a potential in the management of diabetes as well as cardioprotective benefits, but that deserve further clinical investigation [36].

In our study, it is shown that decreased HDL plays an important role in the development of diabetic complications and additional role is played by HDL-associated PON1 enzymes.

**Table 2** Comparison of models 1, 2 and 3

Parameter	Odds ratio	95 % confidence interval
Model 1 ARE (Naglekerke's $R^2$ = 0.593 <i>p</i> value < 0.05)	1.113	1.060–1.168
Model 2 = Model 1 + LACT (Naglekerke's $R^2$ = 0.885, <i>p</i> value < 0.05.)	1.967	1.276–3.032
Model 3 = model 2 + HDLc (Naglekerke's $R^2$ = 0.997, <i>p</i> value < 0.05.)	1.852	1.111–3.087

## Conclusion

To conclude, we can say that cases of type 2 DM patients with vascular complication have reduced PON1 arylesterase and lactonase activity as compared to diabetic without complication as well as normal subjects. Such reduced activity is obtained probably because of its utilization for combating excess oxidative stress in case of persons having type 2 DM with complication. Our study shows PON1 lactonase and PON1 arylesterase combined with HDL-C can predict the development of vascular complication in case of type 2 DM.

## Compliance with ethical standards

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed written consent was obtained from all individual participants included in the study.

**Conflict of interest** The authors declare that they have no conflict of interests.

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ORIGINAL ARTICLE

# Association of TCF7L2 gene variant with T2DM, T1DM and gestational diabetes in the population of Northeastern UP, India

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**Abstract** The non-coding variant (rs7903146) for transcription factor 7-like 2 gene (TCF7L2) is known to be associated with increased risk of type 2 diabetes mellitus (T2DM), but this variant is also associated with type 1 diabetes mellitus (T1DM) and gestational diabetes mellitus (GDM). The association of TCF7L2 variant rs7903146 was confirmed in the Indian and European population for T2DM. We investigated whether TCF7L2 variant rs7903146 is associated with T1DM, T2DM and GDM in Uttar Pradesh population. Three hundred thirty-three patients were genotyped having T2DM, 175 patients with T1DM and 102 gestational GDM and 487 healthy controls. The rs7903146 polymorphism was genotyped using the PCR-based RFLP method. The heterozygous CT genotype of rs7903146 had a 0.0437-fold increased risk in T2DM [OR (95 % CI) 0.0437 (0.0059–0.3213),  $p < 0.00001$ ] and a 0.0081-fold increased risk in T1DM [OR (95 % CI) 0.0131 (0.0011–0.0589),  $p < 0.00001$ ] in comparison to control. The frequency of CT genotype was significantly higher in T2DM than in controls (10.51 vs. 0.62 %) with an OR of 0.0528 (95 % CI 0.0182–0.0189,  $p < 0.0001$ ). The frequency of the CT genotype was significantly higher in T1DM than in controls (38.86 vs. 0.62 %) with OR of 0.0098 (95 % CI 0.0051–0.0497,  $p < 0.0001$ ). The frequency of CT genotype in

T1DM was more than in T2DM. No association was observed in GDM. The study proved that the rs7903146 variant of the TCF7L2 gene is associated with T2DM and T1DM but not GDM in the North Indian population of Uttar Pradesh.

**Keywords** Transcription factor 7-like 2 (TCF7L2) gene · Type 2 diabetes mellitus (T2DM) · Type 1 diabetes mellitus (T1DM) · Gestational diabetes mellitus (GDM) · Genetic association

## Introduction

Diabetes is an inherited non-communicable disease [1] which occurs due to chronic hyperglycaemia or impairment in insulin secretion and action [2]. It is a chronic disease leading to diabetic nephropathy, atrial fibrillation, retinopathy, diabetic foot, neuropathy, coronary vascular disease, hypertension, cholecystectomy, pancreatitis and urinary tract infection. Diabetes can be classified as type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM). T2DM is the most heterogeneous type of disease which occurs due to insulin resistance and reduced insulin secretion [3] with a very high prevalence in comparison to T1DM and GDM.

The high prevalence of T2DM may be due to interaction of environmental and genetic factors. The environmental factors include the increase in food intake and less physical activity. Studies show that Asian Indians are obese [4] and resistant to insulin than Europeans [5], making Indians more susceptible to diabetes [6]. Several studies have been done to check the genetic susceptibility of diabetes with transcription factor 7-like 2 gene (TCF7L2) worldwide.

Santosh K. Yadav, Rashmi, K. K. Tripathi and Royana Singh contributed equally to this work.

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**Table 1** Characteristics of the general feature of population with different subgroup with diabetes (T1DM, T2DM and GDM)

Variables	Healthy control (n = 487)	Type 1 diabetes (n = 175)	Type 2 diabetes (n = 333)	Gestational diabetes (n = 102)
Male/female ratio	1.512:1	1.24:1	1.44:1	Only female
Body mass index (kg/m <sup>2</sup> )				
<25 kg/m <sup>2</sup> normal	385 (79.05 %)	112 (64 %)	205 (61.56 %)	37 (36 %)
25–29 kg/m <sup>2</sup> Overweight	83 (17.04 %)	38 (22 %)	96 (28.82 %)	25 (25 %)
30+ kg/m <sup>2</sup> obese	19 (3.91 %)	25 (14 %)	32 (9.60 %)	40 (39 %)
Treatment				
No treatment	0 %	0 (0%)	0 %	64 (62 %)
Insulin	0 %	52 (30 %)	0 %	27 (26 %)
OHA	0 %	53 (30%)	310 (93.09 %)	0 %
OHA + insulin	0 %	70 (40 %)	23 (6.90 %)	12 (12 %)
Education				
>12 class	34 (6.99 %)	66 (39 %)	246 (73.87 %)	20 (19 %)
Graduation	43 (8.829 %)	85 (50 %)	77 (23.12 %)	19 (19 %)
Post graduation and other higher education	410 (84.18 %)	18 (11 %)	10 (3.03 %)	63 (62 %)
Locality				
Urban	202 (41.53 %)	75 (43 %)	116 (34.83 %)	76 (75 %)
Rural	285 (58.46 %)	100 (57 %)	217 (65.165 %)	26 (25 %)
RBS (mg/dl)	96.242 ± 21.07447	251.3851 ± 152.6732	228.02 ± 101.76	252.5
Food habit				
Non-vegetarian	405 (83.16 %)	86 (49 %)	155 (46.54 %)	48 (47.05 %)
Vegetarian	82 (16.92 %)	89 (51 %)	178 (53.45 %)	54 (52.94 %)
Addiction				
No addiction	365	175	227	102
Tobacco	53*	0	126*	0
Smoking	42*	0	23*	0
Alcohol	36*	0	27*	0

\*In habits, some subjects used to take tobacco, smoking and alcohol; hence, the number was more than 333

TCF7L2 gene is one of the most important candidate genes involved in insulin synthesis and processing [7]. TCF7L2 gene belongs to the class of transcription factor and plays a significant important role in Wnt signaling cascade [8]. TCF7L2 gene by heterodimerizing with β-catenin in β-pancreatic cells, L cells of intestine and in adipocytes is known to show transcription of genes including intestinal proglucan [9]. In the Icelandic, West African, Danish, Polish, Italian and U.S. populations, strong association of TCF7L2 has been shown with T2DM [10, 11].

A strong association of TCF7L2 gene with T2DM has been done in different replicative studies in different Indian populations [12–16]. A study on Indian population from Southwest India and North Indian cohorts has shown strong association of TCF7L2 variants with T2DM [3]. A number of variants are known to be associated with T2DM, though less study has been done on T1DM and GDM.

The aim of the present study was to confirm the role of the common genetic variant rs7903146 C > T, of *TCF7L2* gene in

predisposition to T2DM, T1DM and GDM in the North Indian population of Uttar Pradesh.

## Patients and methods

### Patients

In the present study, patients were enrolled at the Department of Medicine, Institute of Medical Sciences (IMS), Banaras Hindu University, diagnosed with T1DM, T2DM and GDM.

The study was approved by the Institute Ethical committee. Written informed consent was obtained from all the participants.

### Study population

The study involved 333 T2DM patients 175 T1DM patients, 102 GDM and 487 healthy controls. T2DM, T1DM and GDM

**Table 2** Clinical and biological characteristics of study population

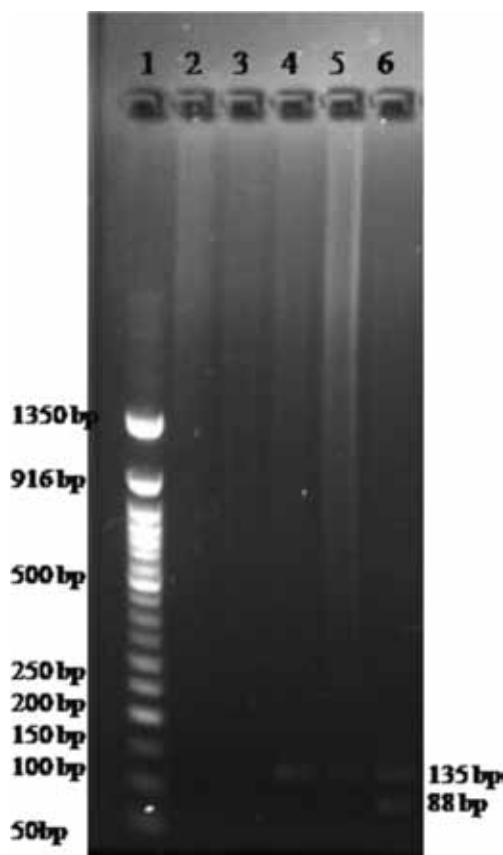
Variables	Healthy control (n = 487)	Type 1 diabetes (n = 175)	Type 2 diabetes (n = 333)	Gestational diabetes (n = 102)	P value (Wilcoxon signed rank test)
Age (years)	28.12 ± 4.515	29.71 ± 14.18	54.74 ± 11.63	30.65 ± 7.009	<0.0001
Systolic blood pressure	119.1 ± 10.88	139.9 ± 17.46	137.8 ± 24.45	127.6 ± 6.576	<0.0001
Diastolic blood pressure	78.81 ± 9.609	83.15 ± 6.357	79.63 ± 11.53	82.92 ± 5.248	<0.0001
Body mass index (kg/m <sup>2</sup> )	20.12 ± 1.24	21.00 ± 3.835	23.99 ± 4.748	28.43 ± 4.184	<0.0001

patients were diagnosed according to the International diabetes federation (IDF) criteria [17]. Non-diabetic controls were recruited from the general population and included in the study after being tested negative for diabetes (fasting plasma glucose <108 mg/dl) [17].

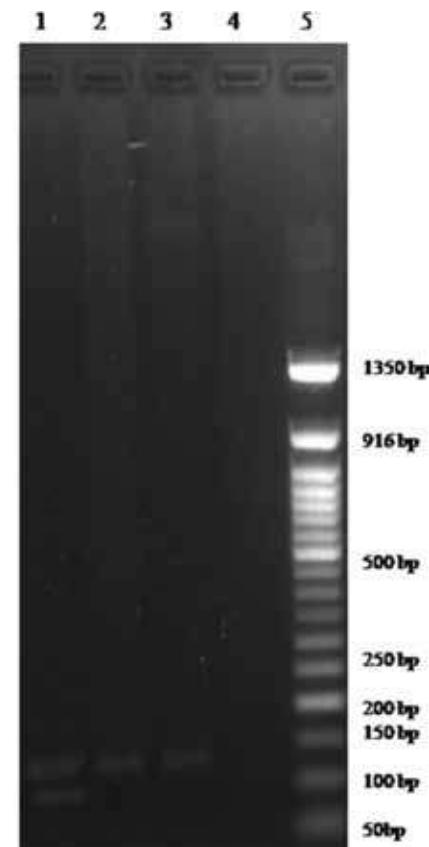
The sex and age, height, waist and hip circumference to the nearest 0.5 cm, and weight in light clothes to the nearest 0.1 kg were measured for all the participants as well as the T2DM, T1DM and GDM. The body mass index (BMI) as weight in kg/height<sup>2</sup> in m<sup>2</sup> was calculated. The resting blood pressures were measured using standardized procedures with an automatic sphygmomanometer.

### Molecular genotyping

Genomic DNA was extracted from whole blood by sodium perchlorate method and stored at -20 °C. The rs7903146 (C/T) polymorphism of the TCF7L2 was genotyped by restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR) using the following primers: forward 5'-GCACAGCTGTTATTACTGAACAAT-3', reverse 5'-ATTGACTAAGTTACTTGCCTCCC-3' (Eurofins Genomics India Pvt. Lts, Bangalore, India). A final reaction volume of 20 µl for the polymerase chain reaction (PCR) was constituted, which contained 100 ng of genomic DNA (1 µl), 5 pM of each primer (1.5 µl), 10 mM of each deoxynucleotide



**Fig. 1** RFLP analysis showing TCF7L2 gene in T1DM patients. *Lane 1*, 50-bp ladder; *lane 2*, negative control; *lane 3*, positive control for TCF7L2; *lane 4 and 5*, showing undigested product; *lane 6*, positive for TCF7L2 mutation, 135- and 88-bp digested products



**Fig. 2** RFLP analysis showing TCF7L2 gene in T2DM patients. *Lane 1*, positive for TCF7L2 mutation, 135- and 88-bp digested products; *lane 2 and 3*, showing undigested product; *lane 4*, negative control; *lane 5*, 50-bp ladder

**Table 3** Genotypic frequency of type 2, type 1 and gestational diabetes in comparison to control group

Genotype	Control		Type 2 diabetes		Type 1 diabetes		Gestational diabetes	
	(n)	%	(n)	%	(n)	%	(n)	%
CC	484	99.38	298	89.49	107	61.142	102	100
CT	3	0.61	35	10.51	68	38.85	0	0
TT	0	0	0	0	0	0	0	0

triphosphate (dNTP) (NEB) (0.5 μl), 5000 U/ml of Taq DNA polymerase (NEB) (0.2 μl), 10× Standard Taq Reaction buffer and 13.3 μl of MQ water.

The PCR was carried out on an Applied Biosystems 96-well VeritiThermal cycler under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s, 72 °C for 45 s and a final extension of 72 °C for 7 min. The amplicon (135 bp) was then digested with a Thermo Scientific (RsaI) restriction enzyme at 37 °C for 16 h. The reaction volume was set to 32 μl, containing 10 μl of PCR reaction mixture (0.1–0.5 μl of DNA), 10× Tango buffer (Thermo Scientific) 2 μl, 2 μl of RsaI and 18 μl of nuclease-free water. The digested products were separated by electrophoresis on a 2 % agarose gel in presence of ethidium bromide (10 mg/ml) and visualized under a UV transilluminator (Biorad). The C allele creates a restriction site and gives two fragments 85 and 50 base pairs after digestion with the restriction enzyme.

## Statistical analysis

Data was analyzed with a Hardy-Weinberg calculator and odd ratio calculator. Genotype and allele frequencies were compared using the  $\chi^2$  statistics or the Fisher's exact test. The Hardy-Weinberg equilibrium was tested using the goodness-of-fit chi-square. Odd ratios were calculated by logistic regression adjusting for age. A *p* value less than 0.05 was considered statistically significant.

## Results

### Characteristics of subjects

The clinical profile of the subjects, represented by mean of different variables such as age of the subjects, BMI,

male/female ratio, treatment if any, educational qualification, food habit, any other habits, random blood sugar (RBS), locality, etc. is presented in Table 1.

The mean age for healthy control was  $28.12 \pm 4.515$ , T1DM was  $29.71 \pm 14.18$ , T2DM was  $54.74 \pm 11.63$  and GDM was  $30.65 \pm 7.009$  which was statistically significant (*p* < 0.0001). The male to female ratio for healthy control individuals were 1.512:1, 1.24:1 in T1DM individuals, and 1.44:1 in T2DM individuals. BMI was calculated for sub-groups, and it was observed that individuals with T1DM showed 14 %, T2DM showed 9.6 % and GDM showed 39 % obese individuals in comparison to 3.91 % healthy control obese individuals. Although treatment was being taken in the form of OHA along with insulin (in 40 % cases), it was observed that the level of random blood sugar (RBS) was very high in T1DM ( $251.3851 \pm 152.6732$ ). The level of SBP ( $139.9 \pm 17.46$ ) and DBP ( $83.15 \pm 6.357$ ) was increased in T1DM, T2DM and GDM in comparison to the healthy control group, and the results were statistically significant (*p* < 0.0001) (Table 2).

### Genotyping study of TCF7L2 in patient and controls

One hundred and sixty six (166) cases were positive for genotyping of T1DM and T2DM, characterized on agarose gel by a single band of 135 bp for the wild-type homozygote CC, and two bands of 135 and 88 bp for the mutant heterozygote CT. Fragments smaller than the 50 bp of the molecular weight marker were not visualized. Hence, the third band of 47 bp was not seen (Figs. 1 and 2).

Genotypic analysis in different types of diabetes (Table 3) showed the frequency of the CC genotype was 89.49 % (298/333) versus 10.51 % (35/333) for the CT and 0 % (0) for the TT genotypes, respectively, in T2DM. The frequency of the CC genotype was 61.142 % (107/

**Table 4** Genotypic analysis of type 2 and type 1 diabetes using the Hardy-Weinberg Principle

Genotype	Type 2 diabetes			Type 1 diabetes			<i>P</i> value
	H-W freq (%)	Allelic frequency (%)	<i>P</i> value	Genotype	H-W freq (%)	Allelic frequency (%)	
CC	89.77	C = 94.74	0.3338	CC	64.92	C = 80.57	0.0014
CT	9.96	T = 5.26		CT	31.31	T = 19.43	
TT	0.28			TT	0		

**Table 5** Association between the TCF7L2 rs7903146 (C/T) polymorphism with type 2 and type 1 diabetes in comparison to control

	Rate	Risk ratio	Odds	Odds ratio	Log odds	95 % Confidence interval	Chi-square Pearson	Fisher <i>t</i> test
Control	0.0062	0.0586	0.0062	0.0528	-2.947	0.0182–0.0189	43.81 (<0.0001)	(<0.0000138)
Type 2 diabetes	0.1051		0.1174					
Control	0.0062	0.0159	0.0062	0.0098	-4.6302	0.0051–0.0497	196.63 (<0.0001)	(<0.0000156)
Type 1 diabetes	0.3886		0.6355					

175) versus 38.85 % (68/175) for the CT and 0 % (0) for the TT genotypes, respectively, in T1DM.

Genotype distribution for rs7903146 was done using the Hardy-Weinberg equilibrium in both cases and controls separately for T21DM and T2DM. Genotype frequencies violated the Hardy-Weinberg equilibrium in the G2DM. The C allele was major with a frequency of 94.74 %, as compared to the minor T allele which showed a frequency of 5.26 % in T2DM. In T1DM, the C allele frequency was 80.57 % and T allele frequency was 19.43 % and was found to significantly increase the risk of T1DM (Table 4).

The carriers of the heterozygous CT genotype of rs7903146 had a 0.0437-fold increased risk in T2DM [OR (95 % CI) 0.0437 (0.0059–0.3213),  $p < 0.00001$ ] and 0.0081-fold increased risk in T1DM [OR (95 % CI) 0.0131 (0.0011–0.0589),  $p < 0.00001$ ], in comparison to control.

The frequency of the CT genotype was significantly higher in T2DM than in controls (10.51 vs. 0.62 %) and was found to be significantly associated with an OR of 0.0528 (95 % CI 0.0182–0.0189,  $p < 0.0001$ ) (Table 5). The frequency of the CT genotype was significantly higher in T1DM than in controls (38.86 vs. 0.62 %) and was found to be significantly associated with an OR of 0.0098 (95 % CI 0.0051–0.0497,  $p < 0.0001$ ) (Table 5). However, the frequency of CT genotype in T1DM was more than in T2DM.

## Discussion

The TCF7L2 gene (rs7903146) polymorphism is a much known single genetic variant influencing T2DM, T1DM and GDM identified to date [17–21]. The present study illustrates that rs7903146 variant of the TCF7L2 gene is associated with a slight risk of T1DM and T2DM in the North Indian population of Uttar Pradesh. The frequency of CT genotype was more in T1DM (38.85 %) than in T2DM (10.51 %). The risk estimates showed 0.0586 in T2DM and 0.0159 increases in T1DM with respect to control. TCF7L2 is a transcription factor and is located in chromosome 10q25.3 [22]. The genetic variant rs7903146 is present in an intronic 92-kb interval having a very strong effect on T2DM. Previous studies have shown that this transcription factor is involved in WNT signalling pathway having a significantly important role in growth and development of a cell [23–28]. TCF7L2 gene was known to be

associated with type 2 diabetes mellitus [29]. TCF7L2 is associated with impaired insulin secretion by its expression in pancreatic beta cell and affects WNT signalling by influencing beta cell proliferation during pregnancy [23, 30].

However, the exact mechanism to confer the process behind diabetes is currently unknown.

This study strongly suggests that the rs7903146 variant of the *TCF7L2* gene is associated with T1DM, T2DM but not GDM in the North Indian population of Uttar Pradesh.

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**Compliance with ethical standards** This manuscript is based on original work and had not been published in whole or part, in any print or electronic media or is under consideration of publication in any print or electronic media other than as abstract of conference proceedings. The study was approved by the Institute Ethical committee. Written informed consent was obtained from all the participants.

**Conflict of interest** The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

# Impact of selected pre-processing techniques on prediction of risk of early readmission for diabetic patients in India

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**Abstract** Diabetes is associated with increased risk of hospital readmission. Predicting risk of readmission of diabetic patients can facilitate implementing appropriate plans to prevent these readmissions. But the real-world medical data is noisy, inconsistent, and incomplete. So before building the prediction model, it is essential to pre-process the data efficiently and make it appropriate for predictive modelling. The objective of this study is to assess the impact of selected pre-processing techniques on the prediction of risk of 30-day readmission among patients with diabetes in India. De-identified electronic medical records data was used from a reputed hospital in the National Capital Region in India and included diabetes patients  $\geq 18$  years old discharged from hospital in 2012 to 2015 ( $n = 9381$ ). This paper focused on data pre-processing steps to improve readmission prediction outcomes. The impact of different pre-processing choices including feature selection, missing value imputation and data balancing on the classifier performance of logistic regression, Naïve Bayes, and decision tree was assessed on various performance metrics such as area under curve, precision, recall, and accuracy. This comprehensive experimental study, first time done from Indian healthcare perspective, offered empirical evidence that most proposed models with pre-processing techniques significantly outperform the baseline

methods (without any pre-processing) with respect to selected evaluation criteria. Area under curve (AUC) was highly increased with the use of oversampling technique as data is skewed on class label Readmission. Recall was the biggest gainer with range increasing from 0.02–0.23 to 0.78–0.85, and there was also an increase in AUC from range 0.56–0.68 to 0.83–0.86 by using pre-processing approach. Data pre-processing has a significant effect on hospital readmission predictive accuracy for patients with diabetes, with certain schemes proving inferior to competitive approaches. In addition, it is found that the impact of pre-processing schemes varies by technique, signifying formulation of different best practices to aid better results of a specific technique.

**Keywords** Data mining · Diabetes · Feature selection · Missing value imputation · Predicting readmission rates · Pre-processing

## Introduction

Diabetes is associated with increased risk of hospital readmission and many times within a short span of time [1]. Many predictive models have been developed for readmissions especially in the USA where hospitals are penalized for unplanned 30-day readmissions. However, not much work has been done in Indian hospitals to predict readmissions because the lack of digitized data serves as a barrier, and there is no incentive resulting from readmission-based payment penalties. But, health insurance companies in India are considering the inclusion of readmission metrics in reimbursement structures and are expected to come up with strong recommendations in the near future [2].

Prediction of risk of readmission is a perplexing task as it requires to integrate various attributes related to patients, such

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as patients' health conditions, socio-demographic factors, and usage of healthcare services. The first biggest challenge is the understanding and identification of the relevant attributes (factors or features) and data values existing in the high-dimensional healthcare dataset that leads to readmission of patients with diabetes. The real-world healthcare data is noisy, inconsistent, heterogeneous, and plagued with a large amount of missing values. So before starting the task of building the model, it is essential to pre-process the data efficiently and make it appropriate for predictive modelling. Lastly, one has to have the profound knowledge of different predictive modelling techniques, so that effective algorithmic solutions could be formulated to solve the prediction problem.

The primary contributions done in this paper can be summarized as follows:

1. By partnering with a leading Indian hospital, embarked on the task of identifying patients with diabetes who were most likely to get readmission within 30 days of discharge for any cause. Formalized the problem and studied the attributes related to cause of readmission for patients with diabetes.
2. To overcome the three main factors that make the data used in readmission prediction task, complex: proposed attribute selection to deal with the high dimensionality of the data, used data imputation to overcome the missing value problem, and used class balancing techniques to solve the problem of skewed data.
3. Conducted an extensive experimental study which used a real-world dataset of about 10,000 medical records that demonstrated the results. Also, this is the first such study done from Indian healthcare perspective.

## Related work

Numerous previous studies have analyzed the risk factors that predict readmissions rates of patients with diabetes [1, 3–7]. Silverstein [3] found that acute and chronic glycemic control influenced readmission risk in a dataset of more than 29,000 patients over the age of 65. Strack [4] studied the impact of HbA1c on readmissions. Eby [5] analyzed the readmission risk for a dataset of more than 52,000 patients in the Humedica network. The most common methods used to predict readmission are stepwise logistic regression [8, 9] and multivariate logistic regression [10, 11]. And the most common technique to evaluate the model performance is to use receiver operating characteristic (ROC) curve, AUC (area under the curve), and C-statistic [10–12]. Most common predictive models are LACE [13], PARR [14], and KEN [15]. These models are difficult to implement in an Indian healthcare scenario as little digitization of health data is done

in Indian hospitals and rare use of International Classification of Diseases (ICD) codes. Meadem [16] has done a similar study on exploring pre-processing techniques for prediction of risk of readmission for congestive heart failure patients. This study further enhances Meadem's findings by considering a different disease and a real-world dataset from an Indian hospital. Consequently, results are more reflective of the problem of readmissions among patients with diabetes in the Indian context. The enormous growth of the digital health data (called Big Data) generates opportunities for greater patient insights that may help lower healthcare costs in India while providing better healthcare access [17, 18]. This proposed solution is scalable to any volume, variety, and velocity of data resulting in more efficient and faster results and insights.

## Proposed method

The overall framework of the proposed method is depicted in Fig. 1.

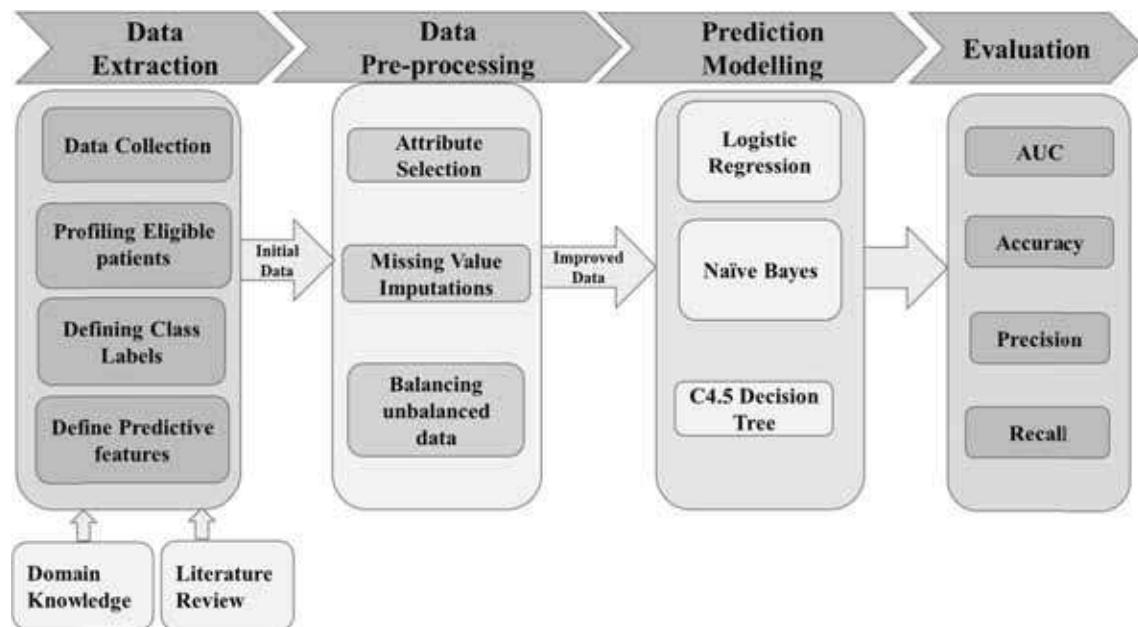
### Data extraction, integration, and exploration

This phase explored the raw data to discover initial insights and recognize interesting actionable patterns. In this work, a dataset of 9381 diabetic patient encounters was extracted from their electronic medical record (EMR) system, over a period of 2 years (2013–2015) from an Indian hospital. Before analyzing the data, all patient records and information were anonymized and de-identified. Continuity of patient encounters within the same health system was preserved. The necessary knowledge of the medical domain was acquired to investigate the dataset using valuable inputs from medical experts.

#### Data selection

The following criteria were applied to extract the necessary dataset for patient visits:

1. It was an inpatient hospital admission.
2. Length of stay (LOS) is minimum 1 day. Day care not included.
3. Patients above 18 years.
4. Excluded encounters due to giving birth/delivery because such encounters either are pre-scheduled or inevitable.
5. It was a hospital visit by a patient with diabetes, that is, one during which any kind of diabetes is entered into the system, as a diagnosis or in past history.



**Fig. 1** The overall architecture of the Hospital Readmission Risk Prediction modelling process

A total of 9381 encounters were identified to fulfill all of the above five inclusion criteria and are used in further analysis.

#### Defining class labels

The class label was defined as a dichotomous variable which was assessed taking into account whether a patient was readmitted within 30 days of discharge. Since the primary interest was in factors that lead to early readmission, the readmission attribute (outcome) was defined as having two values: *Yes* if the patient was readmitted within 30 days of discharge or *No* if otherwise, which covered both readmission after 30 days and no readmission at all. The readmissions considered here were all-cause readmissions. As shown in Fig. 2, out of total 9381 records, 1211 (12.9 %) encounters were found as readmissions within 30 days of discharge from the hospitalization.

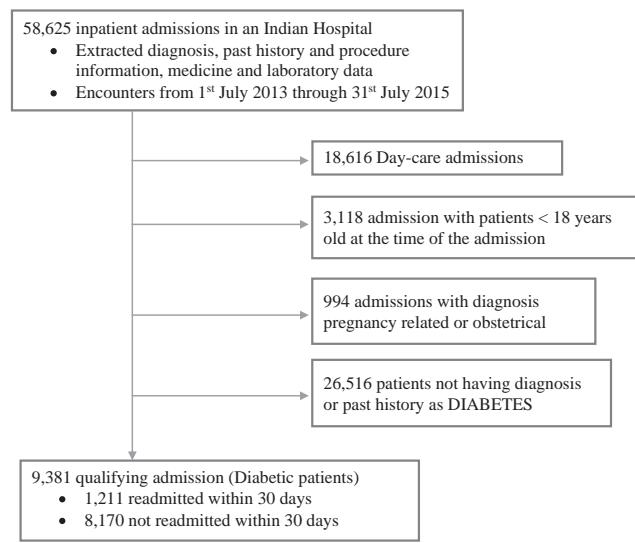
#### Define predictive features

Several brainstorming sessions with domain experts from a reputed Indian hospital were conducted. Through an extensive review of related studies [1, 4–7, 16, 19] and the help of domain experts, only attributes that are potentially associated with the diabetic condition or management influencing early readmissions were retained. Figure 3 lists the features which were used in building predictive models (italic features are derived features). The variables chosen for patient demographic and illness severity were gender, age, admission source, and time spent in the hospital. Comorbidities,

abnormal lab results, and prescription drugs related to diabetes were also included to gauge the acuity of patient's condition.

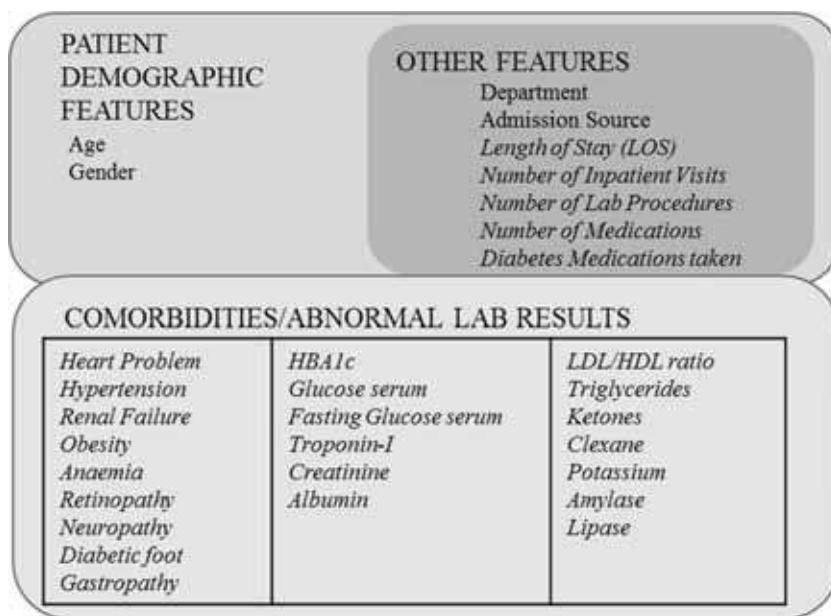
#### Data pre-processing

The following pre-processing techniques were applied to refine the data further. These are some of the popular techniques used for readmission prediction. Feature selection is chosen to deal with the high dimensionality of the data [16, 20, 21]. Data imputation is used to overcome the missing value problem [16, 22, 23], and class



**Fig. 2** Flow chart showing inclusion and exclusion of inpatient admissions in the analytic cohort

**Fig. 3** Features used in the risk of readmission prediction process



balancing techniques are used to solve the problem of skewed data [16, 23, 24, 25].

**Feature selection** One of the real challenges before the classification task was to identify the subset of attributes that significantly impact readmission of patients from the numerous attributes present in the data set. Two state-of-the-art feature selection techniques were considered: correlation-based filter approach and Pearson's chi-square test [26, 27].

**Missing value imputation** It was observed that some of the important attributes in the dataset have no value for certain patients. These missing values not only impede the actual prediction task but may also lead to biased results. This paper used a simple but effective mean/mode imputation (MMI) technique for imputing missing values [28, 29]. MMI fills in missing data with the mean for the numeric attribute or with the mode for the nominal attribute of all cases observed.

**Reducing class imbalance** After data integration, in most of the cases, high skewness was observed in the labelled dataset. In this case, it meant that the number of instances with *No* for Readmission class label (8170 records) significantly outnumbered the number of instances with *Yes* for Readmission class label (1211 records). Such imbalance introduces biasness in the actual predictive model. The reason being the model having such skewed class distribution would certainly predict the majority class as a class label far more frequently than the minority class. To circumvent that problem, both oversampling (OS) and under-sampling (US) techniques were used which altered the class distribution of the

training dataset in such a way that both classes were well represented.

### Predictive modelling

For the final classification task, three predictive models were used.

**Logistic regression (LR)** It is a statistical model that attempts to fit a line to the data. Rather than fitting the line directly to the binary outcome, it uses a logit function of the predictive variables which is a transformation of the outcome [30].

**Naïve Bayes (NB)** It calculates the probability of a given record belonging to a particular class. It assumes that given the class, features are statistically independent of each other. This assumption is called as class conditional independence which greatly simplifies the learning process [26].

**Decision tree (DT)** It constructs a hierarchical tree-like structure, and its goal is to create a model that predicts the value of a target variable based on several input variables. Here, C4.5 statistical classifier is used which is a popular classifier and prediction method for handling high-dimensional data. It decides the most significant independent variable in each stage of predicting dependent variables [31].

Many other classifiers were available, but as the main objective of this work was to showcase the effect of pre-processing techniques and not to compare classifier performances, this study limited itself to these three classifiers.

## Experimental evaluation

The experiments were conducted using the tool WEKA v3.6.12 [32] to develop the models and MySQL v6.3 for the database. The dataset for this research held approximately 7100 patients diagnosed with diabetes and servicing over 9300 hospital encounters. Data was provided by the hospital in Excel sheets extracted from their EMR system. After further exploration of the dataset, review of related studies, and with the help of domain experts, 31 attributes were identified to be related to the risk of diabetic patients' readmission, out of which 27 were derived attributes (*italic* in Fig. 3). Testing of the predictive model was done by using 10-fold cross-validation procedure. MMI was performed using tool WEKA v3.6.12. Four evaluation metrics were considered to assess the quality of the different models:

1. Area under the curve (AUC): This measure evaluates the trade-off between the rate of patients that are correctly classified as *Yes* for Readmission class label and the rate of patients incorrectly classified as *Yes*.
2. Precision: This is the probability that a patient that is predicted as *Yes* for Readmission indeed belongs to the class *Yes* for Readmission.
3. Recall: This measures the probability that a patient that truly belongs to the class *Yes* for Readmission is also predicted to be *Yes* for Readmission.
4. Accuracy: This is the rate of correctly classified patients.

Based upon the final objective of the readmission risk prediction, the different measures of evaluation are more or less suitable. The AUC measure is typically interesting when the problem is imbalanced as stated previously in this case. The precision is the proportion of predicted positives which are actual positive. It is relevant when there is a high cost associated with falsely predicting patients to fall in the class *Yes* for Readmission, when actually they are not. Recall is important if the identification of patients which fall into the category of *Yes* for Readmission class label is the main objective. The accuracy is the proportion of the total number of predictions that were correct. It is the standard evaluation measure that provides a global insight in the performance of the model. In this work, a baseline model was developed first for each predictive model—LR, NB, and DT C4.5 which used no pre-processing technique. After this, data pre-processing techniques were applied on predictive models and the models were evaluated based on AUC, precision, recall, and accuracy. Two feature selection techniques were considered and evaluated using tool WEKA v3.6.12. The first one was correlation-based feature selection evaluation criteria which evaluated the importance of subsets of features that were highly correlated with the class while having low inter-correlation. The

second one was chi-squared attribute evaluation which evaluated the importance of an attribute by computing the value of the chi-squared statistic with respect to the class.

## Results and discussion

The results are presented in Table 1. In the first three columns, it is indicated which pre-processing techniques were applied. In the fourth column, the predictive model is shown and the last four columns show the resulting evaluation metrics. For brevity, only the models that were in the top three with respect to performance are shown for the considered evaluation metrics. Each line in the table represents a model and it is indicated which pre-processing algorithms were applied. A minus sign means that none of the corresponding pre-processing techniques is carried out.

As noticed in Table 1, the following observations have been made:

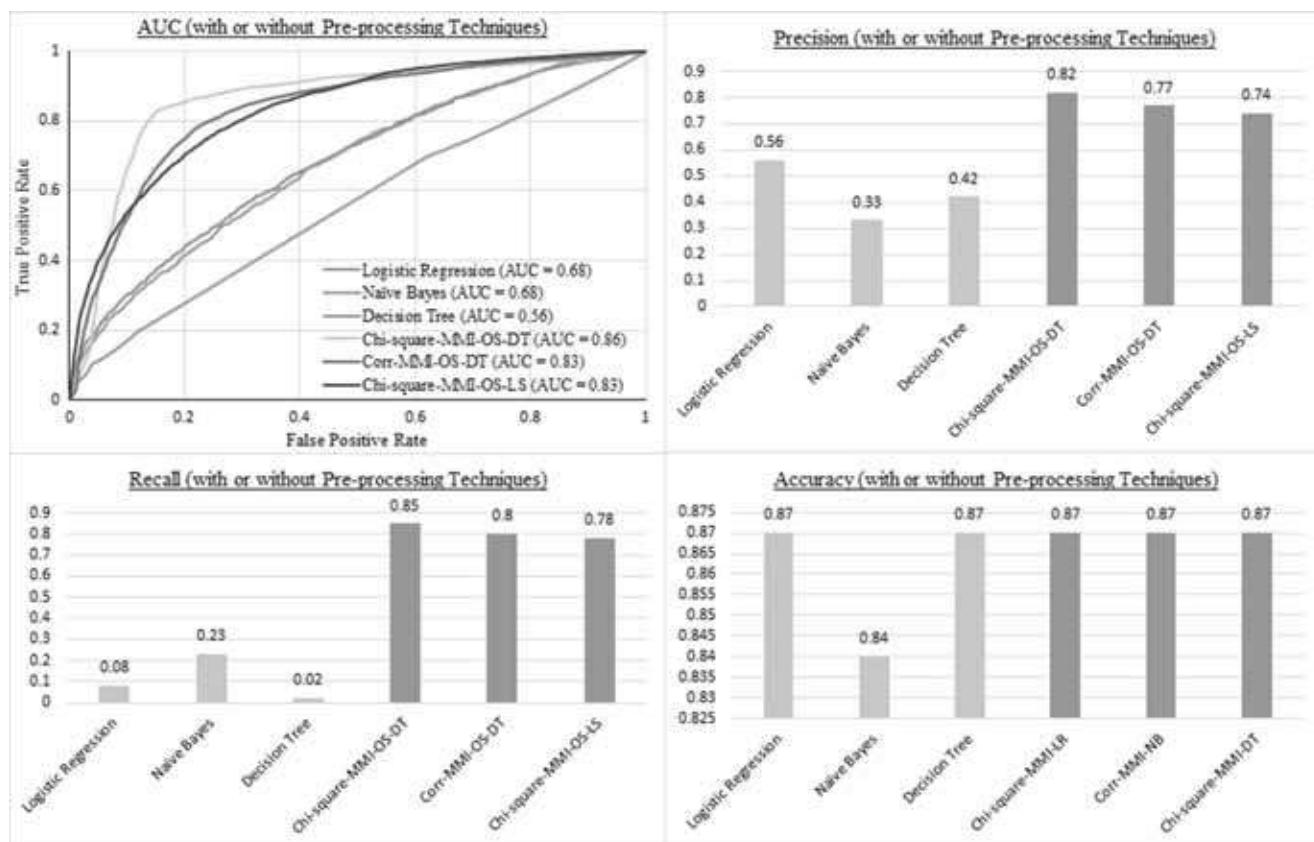
- All proposed models chosen in this study, with selected pre-processing techniques, significantly outperform the baseline methods. It is observed that the best predictive model in this study is decision tree performed with chi-square feature selection along with MMI and oversampling pre-processing techniques.
- The baseline methods using decision tree (DT) or logistic regression (LR) as predictive model are outperformed for AUC, precision, and recall by the new method that uses feature selection, mean/mode data imputation (MMI), and oversampling (OS) pre-processing techniques as shown in Fig. 4.
- AUC is highly increased with the use of oversampling technique from range 0.56–0.68 to 0.83–0.86 as data is skewed on class label Readmission.
- Naïve Bayes prediction model is least positively affected by the use of pre-processing techniques if compared to decision tree or logistic regression.
- Recall is the biggest gainer and has the biggest improvement for all predictive models with range increasing from 0.02–0.23 to 0.78–0.85 after applying pre-processing techniques.
- Accuracy has slight improvement only for Naïve Bayes model after applying feature selection and missing value imputation techniques. The reason being, as the given data set is unbalanced, the classifier is biased towards majority class yielding a very optimistic accuracy estimate already. No pre-processing technique has much effect on improving accuracy.
- The potential beneficial effects of using pre-processing techniques depend upon the evaluation metric used to assess the performance of the classifier.

**Table 1** Evaluation of the baseline method and the newly proposed techniques

Feature selection	Missing value imputation	Balancing	Predictive model	AUC	Precision	Recall	Accuracy
Baseline							
—	—	—	Logistic regression	0.68	0.56	0.08	0.87
—	—	—	Naïve Bayes	0.68	0.33	0.23	0.84
—	—	—	Decision tree	0.56	0.42	0.02	0.87
Top 3—AUC, precision, and recall							
Chi-square	Mean/mode imputation	Oversampling	Decision tree	0.86	0.82	0.85	0.83
Correlation-based	Mean/mode imputation	Oversampling	Decision tree	0.83	0.77	0.80	0.78
Chi-square	Mean/mode imputation	Oversampling	Logistic regression	0.83	0.74	0.78	0.75
Top 3—accuracy							
Chi-square	Mean/mode imputation	—	Logistic regression	0.68	0.56	0.08	0.87
Correlation-based	Mean/mode imputation	—	Naïve Bayes	0.66	0.56	0.10	0.87
Chi-square	Mean/mode imputation	—	Decision tree	0.58	0.48	0.04	0.87

The research papers by Meadem [16] and Zolfaghari [33] reports very similar methods and results. The AUCs reported by Meadem and most other studies in this area remain 0.6 to 0.7. This study reports an increase in model performance (AUC) from range 0.56–0.68 to 0.83–0.86 by using a similar pre-processing approach. These results are also in-sync with a similar research study done by Chin [34] for readmission prediction using discretization pre-processing technique.

These observations conclude that it is indeed useful to apply pre-processing techniques to the data before creating a readmission prediction model for diabetic patients in an Indian healthcare scenario also. But there is a need to evaluate the medical relevance of the evaluation metric before the application and choice of the pre-processing techniques considered in this work, because all evaluation metrics would not be expected to be of equal medical relevance.

**Fig. 4** Evaluation criteria performance comparison using pre-processing techniques

## Conclusion and future work

Partnering with an Indian hospital, this paper has undertaken the task of describing how various data pre-processing techniques may impact the results of prediction modeling using readmission for patients with a diabetes diagnosis as the context for the analysis. The problem of predicting the risk of readmission was framed as a binary classification problem, and several available prediction models were developed and evaluated. This work explored a complex, high-dimensional clinical dataset provided by an Indian hospital towards identifying the risk factors related to readmission of patients with diabetes within 30 days of discharge. Improvement is done in the predictive ability of the dataset by applying feature selection, missing value imputation, and class balancing techniques. Then, various predictive models like logistic regression, Naïve Bayes, and decision tree were applied to this improved dataset to obtain risk of readmission predictions. The impact of different pre-processing choices was assessed on various performance metrics like area under curve (AUC), precision, recall, and accuracy using the real-world healthcare dataset (around 10,000 patient records). This study offers empirical evidence that most proposed models with selected pre-processing techniques significantly outperform the baseline methods (without any pre-processing) with respect to selected evaluation criteria. AUC is highly increased with the use of oversampling technique if data is skewed on class label. Recall was the biggest gainer with range increasing from 0.02–0.23 to 0.78–0.85, and there was also an increase in AUC from range 0.56–0.68 to 0.83–0.86 by using pre-processing approach. Thus, this study concludes that the impact of pre-processing schemes varies by technique, signifying formulation of different best practices to aid better results of a specific technique.

The authors' ongoing work involves investigation of additional features and classification techniques to improve the quality of readmission prediction for patients with diabetes. They are also in the process of building a predictive model for risk of readmission for other diseases from an Indian healthcare perspective where very little work has been done for identifying major risk factors of readmission.

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## Compliance with ethical standards

**Human and animal rights** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

# Phylogenetic and promoter analysis of islet amyloid polypeptide gene causing type 2 diabetes in mammalian species

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**Abstract** Mutation in islet amyloid polypeptide (IAPP) gene results into its protein misfolding and fibril formation. Isolation of various mammalian species shows its conservation from 89- to 93-amino acid sequence sharing homology with cats, rats, mice, and guinea pigs. For the present study, detailed phylogenetic analysis is carried out for the upstream promoter region of IAPP gene, IAPP messenger RNA (mRNA), and proIAPP protein of nine mammalian species. Sequence analysis has shown partial conservation among the rodent species and the canidae group; however, the primate group species has shown maximum conservation of all sequences under considerations with respect to the human IAPP sequences. Our novel approach of analyzing mRNA and protein sequence conservation of proIAPP delineates the importance for developing IAPP models in closely related species sharing a common ancestor with the time divergence. Further, transcription factor binding sites were critically analyzed for the upstream promoter region of the IAPP gene. These probable binding sites were predicted for DNA motifs recognized by transcription factors, which may prove to be helpful in predicting the regulatory mechanism in understanding the regulation of IAPP gene under in vitro or in vivo conditions. Therefore, confirming the co-evolutionary relationship of IAPP among different species will help us guide in studying transcription factor binding site for poorly studied IAPP gene and targeting disease mutation initiating pancreatic

amyloidosis in clinical applications. The phylogenetic and gene promoter analysis presented in the paper reveals regulatory elements for beta cell death caused by pancreatic amyloidosis and resulting in type 2 diabetes (T2D) onset.

**Keywords** Islet amyloid polypeptide · Type II diabetes · Phylogenetic analysis · Promoter · Transcription factors · Transcription factor binding sites

## Introduction

The analysis of interaction between transcription factor (TF) and its binding site at promoter region is essential for gene regulation studies. Any mutation observed in either two can weaken their interaction and thus drive changes in transcription and translational processes, which may result in metabolic inactivity, and, hence, causing disease onset followed by its progression. To maintain such vital interaction during the course of evolution, the compensatory mutations are observed in the gene regulatory regions. Highly penetrant mutations in gene encoding proteins causing amyloid formation are one of the life-threatening conditions observed due to deposition of abnormal protein in organs. Fragments produced due to misfolding leads to a poorly functioning protein. The fibrils produced are not dissolved by proteolysis and aggregate to form oligomers of  $\beta$ -pleated sheets, which are extremely hydrophobic. Aggregations of these fibrils are toxic to the cell and can interfere with proper organ function. Like many transcriptionally regulated abnormal proteins, islet amyloid polypeptide (IAPP) is a major protein component of amyloid deposits produced in islets of Langerhans of humans with type 2 diabetes (T2D) and other mammalian species [1–3]. This amyloid polypeptide is produced in and stored in the pancreatic islet  $\beta$ -cell and co-

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secreted with insulin [4]. Spontaneous aggregation of human IAPP (hIAPP) into  $\beta$ -sheet-rich amyloid fibrils in patients causes deleterious effects on pancreatic cells. The aggregation is due to misfolding of amyloid fibrils due to mutations observed in IAPP gene regulation. Similar aggregation of misfolded amyloid proteins into amyloid fibrils is found in neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's. Further, it has also been hypothesized that IAPP may enter the brain contributing to A $\beta$  misfolding [5]. The role of IAPP in pathogenesis of T2D is evident from aging individuals identified for insulinomas [6, 7]. In response to glucose or nonglucose stimulants, IAPP synthesized from proIAPP to IAPP [8–10]. The physiological consequences of IAPP deposition include carbohydrate metabolism, modulation of insulin secretion, inhibition of bone reabsorption, suppression of gastric emptying, and food intake [11, 12]. Amyloids formed due to overexpression of IAPP gene follow misfolding of the IAPP itself during post-translational modification. This leads to fibril formation, which further interacts with plasma membranes depending on the concentration of lipids causing beta cell death [13, 14]. Over 40 % of T2D autopsy cases have amyloid deposits associated with the loss of  $\beta$ -cells [15]. Understanding of the genome-wide association studies (GWAS) revealing associations of IAPP with T2D and their genetic polymorphisms arise affecting the overall metabolic pathway of IAPP deposition [16]. However, many pathways and physiological characterization of IAPP still remain elusive in patients and its correlation with T2D.

The emergence of complex, multicellular organisms is accompanied by divergence and dramatic increase in the complexity of gene regulatory mechanism. The divergence in these gene sequences contributes in evolution of speciation and disease progression in different species. Despite the evolution of gene regulation, many animals still possess a core set of gene sequences regulated by TF that were inherited from a common ancestor >500 million years ago; however, variation is also observed in the transcriptional regulation at the promoter region, which is the main cause of variation in disease onset observed among species. Therefore, to delineate the disease onset and its progression, targeting gene regulation at promoter region becomes relevant as it specifies the functionality of the protein and cellular networks that it further targets. In this context, the structural complexity of IAPP holds tremendous variation, with high variability found among the mammalian species. Humans, monkey, and cats are amyloidogenic and also develop T2D. However, the rodent species do not develop T2D as cell line culture studies have revealed non-amyloidogenic to be non-toxic [17], but T2D rodent models have been developed through gene modifications using knock-in and knock-out strategies [18], and many others show relatedness to complex physiological human systems [19] for various diseases such as Down syndrome, type 1 diabetes, cystic fibrosis, cancer, etc. [20–22].

The protein-DNA interaction of IAPP gene regulates various regulatory pathways and cellular mechanisms, further inducing IAPP formation with respect to insulin. However, regulation and misfolding of the IAPP protein can be studied through genetic modifiers which recognize *cis*-regulatory elements or transcription factor binding sites (TFBS). TFs known to regulate IAPP gene are PDX1 $\alpha$ , mediated through A element-like *cis*-motifs in the gene promoter and glucose levels [23, 24]. PAX4, on the other hand, binds to 138 downstream promoter region and also a negative element that exists between sites -111 and -102 [25, 26]. Since the HNF TF families are known to be involved in maturity-onset diabetes of the young (MODY) family members, HNF-3 $\beta$  (FoxA2) negatively regulates the IAPP promoter, while the MODY3 TF HNF-1 $\alpha$  positively regulates IAPP promoter activity [27]. CREB protein has shown direct binding to the CRE-like sequence, which is a pleiotropic element crucial for basal transcription, the cAMP response, and the glucocorticoid response [28]. Although the promoter sequence analysis through protein-DNA binding studies, involving luciferase activities, EMSA, and ChIP analysis, has shown various regulatory binding elements, the role of these elements on the transcriptional regulation of IAPP pathway still remains poorly understood.

In the present study, different parameters are evaluated involving messenger RNA (mRNA) and protein sequence homology of IAPP. The analysis of upstream promoter region, mRNA, and human proIAPP (hpIAPP) sequence homology is carried out among nine mammalian species namely *Homo sapiens* (humans), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Cannis lupus familiaris* (dog), *Felis catus* (cat), *Gorilla gorilla gorilla* (gorilla), *Macaca mulatta* (monkey), *Pan troglodytes* (chimpanzee), and *Sus scrofa* (pig). Further, analysis of the upstream promoter region of IAPP gene identifies probable functional key elements involved. This study clearly demonstrates the species closeness and the specific regions to be taken into account to develop IAPP-specific successful cell culture or animal models. The key elements highlighted can further reiterate pathways, which are important in studying IAPP-induced T2D due to pancreatic amyloidosis.

## Materials and methods

### Data retrieval

hIAPP upstream promoter region [29] IAPP messenger RNA (mRNA) and pro-IAPP protein sequences of nine mammalian species are tabulated as known in literature. The presented data is downloaded from the GeneBank database using the Entrez data retrieval tool (<http://www.ncbi.nlm.nih.gov/Entrez>).

The consensus sequences were retrieved as given in Table 1.

The protein sequences are searched against the human genome database using blastn (<http://blast.ncbi.nlm.nih.gov/>) for the abovementioned data [30]. Genes and protein sequences are named to the convention used in NCBI for genome database.

### MSA and phylogenetic analysis of taxa

The hpIAPP sequence is used as a reference template sequence to study other eight mammalian species. All sequences are compiled using computational model, ClustalW, with default alignment parameters to align protein sequences obtained by blastn. All nine sequences are aligned using blastn (<http://blast.ncbi.nlm.nih.gov/>) for promoter as well as mRNA sequences using ClustalW for protein sequences. Pairwise identities were carried out for all sequences. The process is followed by phylogenetic analysis employing all pro-IAPP protein sequences aligned by multiple sequence alignment (MSA).

### Maximum parsimony trees

An unrooted phylogenetic tree reflecting the distance relationship among all species is constructed using the Mega 6.0 [31] package, which is based on neighbor-joining (NJ) method [32] exploiting the Poisson method [33] with a scale length of 0.05. The tree topology of IAPP is used to assess and match the nine sequences after removing gap-containing positions.

### Molecular clock test

The molecular clock estimation is performed with maximum likelihood method to quantify the time of divergence among the species, calculated for the putative protein sequences using Mega6.

### Transcription promoter analysis

The putative promoter of hIAPP gene is analyzed for transcription factor binding motifs (TFBM) by using the promoter scan program (<http://www.tfbind.hgc.jp>). This analysis also includes the already identified TFBMs from previous studies, which are excluded in the present study. The new probable functional binding site positions present in the promoter region of hIAPP gene are represented here.

## Results

Comparative genome analysis among different species is a powerful method for detecting functional regulatory

**Table 1** Gene accession of the IAPP promoter region

Mammalian species	Gene accession number	Exon
<i>Homo sapiens</i>	NC_000012.12 NP_000406.1	Exon 2—X55634.1 Exon 3—X 52819.1
<i>Mus musculus</i>	NC_000072.6 NP_034621.1 NM_010491.2	
<i>Rattus norvegicus</i>	NC_005103.4 EDM 01507.1 XM_006237614.2	
<i>Cannis lupus familiaris</i>	NC_006609.3 NP 001003233.1 NM 001003233.2	
<i>Felis catus</i>	NC_018729.2 NP 001036803.1 NM 001043338.1	
<i>Gorilla gorilla gorilla</i>	NC_018436.1 XM 004052900 XM_004052850.1	
<i>Macaca mulatta</i>	NC_007868.1 XP_001098180 XM 001098180.2	
<i>Pan troglodytes</i>	NC_006479.3 XP_001144800.2 XM 001144800.2	
<i>Sus scrofa</i>	NC_010447.4 XP 003126485.2 XM 003126437.3	

sequences especially at promoter region. Since TFBS are many a time small in size and highly degenerate, they can, however, reveal important insight into multispecies comparison, which can identify sites that are of functional importance, as these sites are under selective pressure [34, 35]. The following results confirm the co-evolutionary relationship of IAPP promoter region, mRNA sequence, and protein among different mammalian species.

### Conservation of upstream promoter region of IAPP gene

In this paper, the sets of nine mammalian upstream sequences, likely to contain transcriptional regulatory sequences regulating the promoter region of IAPP gene, are assembled. The upstream sequence sets encompass 2817 bp, which are aligned with the orthologous sequence using blastn. The sequence identity of the primates shows high conservation among all species. Maximum conservation of hIAPP upstream promoter region is seen with that of chimpanzees with 98 % sequence identity and *E* value of 0.0. Gorillas and monkeys also aligned the most with a sequence identity of 97 and

91 %. However, mice and rats were found to align least with the upstream promoter region of hIAPP gene with a sequence identity of 64 and 82 %. The 82 % sequence identity alignment between human and rat is attributed to the fact that only 35 % identity is covered, and the promoter data might not exist in genome or may be missing. This may be due to the fact that mice and rat do not develop in context of T2D. The pairwise alignment of human-dog and human-cat shows sequence identity of 75 and 77 %, respectively, indicating high assembly of the datasets and similarity of the promoter region found among the three species. These results clearly prove that the upstream promoter region of IAPP gene is closely associated with that of the primates than the conidae and lower chordates such as the rodents (Table 2).

#### **Conservation of IAPP mRNA sequence among mammalian species**

The upstream promoter region of the hIAPP gene is found to be highly conserved among primate species only. It signifies that the other species might not have sequence conservation encoding exons. Further, the exons 2 and 3 of the mRNA sequences are aligned. Since exon 1 of hIAPP is a non-coding sequence (Fig. 1), maximum alignment percentage is seen between human-chimpanzee, gorilla, and monkey (*E* value 0.0, 0.0, and 1e–94, respectively). This is also consistent with the results of upstream IAPP promoter region stating that the mRNA sequence is highly conserved among the primate species, which are more prone to develop T2D (Table 3).

#### **Sequence conservation of human protein IAPP sequence among mammalian species**

Amino acid sequence of human protein (hpIAPP), retrieved from the data assembly, is compared with that of mammalian species. Rodent species have not shown to develop diabetes or amyloid deposits. Further, proIAPP sequence alignment is carried out. Although the mRNA sequence is not found to be conserved among all species, a possibility of sequence

conservation due to post-translational modification might lead to sequence conservation among various species. The aa sequence conservation is found to be only 48.7 % among all nine mammalian species, whereas 98 % sequence conservation is found among primates (Fig. 2). It can be inferred from the study that mentioned species may be an evolutionary distinct group. The presented inferences confirm that the primate group species are prone to develop IAPP fibrils, as they are at a higher risk to T2D than the rodent species.

#### **Phylogenetic analysis and molecular estimates and constant rate of evolution of hpIAPP**

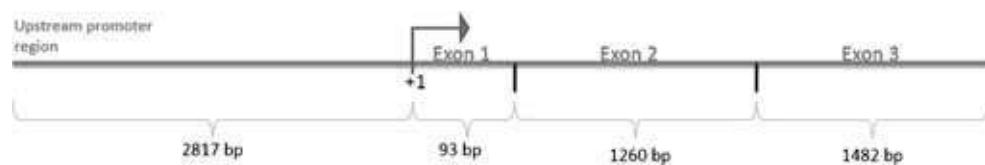
Evolutionary distance among the mammalian species for proIAPP is determined using the neighbor-joining method. It uses distance matrix to construct the phylogenetic tree. Smaller distances among species clearly indicated their close evolutionary relationship and conservation of proIAPP homology. Maximum clustering is found among humans, chimpanzees, gorillas, and monkeys. All primate group species are observed to have close evolution of proIAPP protein. Mice, rats, and pigs show maximum branch length of 0.2 with maximum divergence from other species indicating fast evolution among all taxa. Dogs and cats, on the other hand, show closeness to the primate group with branch lengths of 0.09 and 0.06, respectively, which specify their faster divergence from the primate group (Fig. 3).

Molecular clock estimation is performed to prove divergence of time among the taxa. To analyze evolutionary rate between proIAPP and hpIAPP, the distance matrix plotted against time is evaluated using molecular clock test based on maximum likelihood method (Table 4) (the linear relation must exist for constant rate of evolutions). Followed by data cleaning using elimination of gap and missing data positions, the logarithmic ( $I_o$ ) likelihood values under the clock and no-clock models are calculated to be  $I_o = -932.524$  and  $I_o = -733.232$ , respectively. The molecular test shows that the evolutionary rate is not constant over time among all the mentioned species.

The phylogenetic analysis of the proIAPP amino acid sequence shows 48.7 % sequence conservation among the taxa and maximum conservation of 98 % among the primates. Pairwise distance (p-distance) among the nine sequences is also computed (Table 5) using the Poisson correction model. The primate species does not show any variation in the evolution of the proIAPP sequence. Hence, it can clearly be inferred that the evolutionary rate among the primate pIAPP is constant. However, the maximum pairwise distance is observed only among the rodent species with that of the hpIAPP and followed by the conidae species. The p-distance is calculated for the amino acid distance showing multiple substitution events in the same position as the evolutionary time elapsed.

**Table 2** hIAPP upstream promoter sequence identity and alignment with mammalian species

Species sequence alignment	Sequence similarity (%)	<i>E</i> value
<i>Homo sapiens-Mus musculus</i>	64	2e–19
<i>Homo sapiens-Rattus norvegicus</i>	82	2e–09
<i>Homo sapiens-Canis lupus familiaris</i>	75	0.0
<i>Homo sapiens-Felis catus</i>	77	0.0
<i>Homo sapiens-Gorilla gorilla</i>	97	0.0
<i>Homo sapiens-Macaca mulatta</i>	91	0.0
<i>Homo sapiens-Pan troglodytes</i>	98	0.0
<i>Homo sapiens-Sus scrofa</i>	77	2e–97



**Fig. 1** Human IAPP mRNA with exons 1, 2, and 3. Arrow indicates the transcription start site with *left side* representing the upstream promoter region and *right side* representing the downstream promoter sequence

### Transcriptional binding motifs at the upstream promoter region of hIAPP gene

The regulation of gene expression depends on the TF and enhancer elements present upstream of a promoter region. hIAPP was analyzed for the upstream promoter elements for TF binding motifs. Hits of scanning results allowed to predict new regulatory sequences present upstream of the hIAPP promoter region. Therefore, the new putative regulatory sites are predicted within the upstream region (Table 6). Although many previously known motifs are also evolved, a thorough analysis reveals many unknown TF motifs, which still remain to be elucidated. The TFs identified for the hIAPP gene are scrutinized on the basis of their major role in pancreatic metabolism. Sequence motifs are present on the forward as well the reverse strands, with one TF having multiple sequence at different position as given in Table 6.

Maximum hits (251) and a similarity score of 0.9 (0.0–1.0 between registered sequences for TF binding site) are obtained for Oct1 TF. Other notable and ubiquitous TFs having putative binding site on IAPP promoter region and hits above 50 are GATA1/2, STAT, MEF, AP4, and SP1. Putative binding sites predicted for hIAPP also have known functional consequence of pancreatic metabolic pathways (as compiled in Table 5). Further, wet lab experiments can be useful to confirm whether the proposed binding sequences are functional. Conservation analysis of these binding motifs is also done to predict whether these motifs are evolutionary conserved among the primate groups. The proposed analysis pointed out that the important

regulatory sequences are conserved throughout the primate taxa (data not shown).

### Discussion

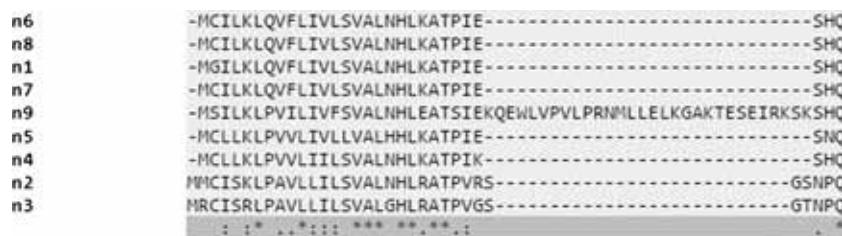
*Cis*-regulatory elements play an important role in gene expression both at transcription and at translational levels. Mutations in regulatory sequences present in the promoter region are pre-eminent in the evolution of TF-regulated gene regulatory networks [63]. That is why it becomes important to study the *cis*-regulatory element sequence, as this is the only variation that has shown how protein expression varies among different species and their divergence over time. Taking humans into account, this comparison of evolutionary conserved sequences delineates important metabolic pathways which can be studied in species and helps produce animal models for advent of detailed assays and analysis of diseases which cannot be carried out in human patients. It further becomes important to determine whether changes in expression pattern have typically occurred or not for the diseases under consideration before invention of therapeutic interventions. Therefore, the significance of studying the phylogenetic relationship among different mammalian species can help target specific DNA sequences in humans and animals. Identifying IAPP gene regulatory elements can now be targeted to promote the development of IAPP-induced T2D animal model. This can give an in-depth analysis of the information that can help correct and rescue disease mutation in clinical applications since IAPP gene regulation and its conservation among species remain elusive till date.

The protein involved in the study is IAPP, specifically proIAPP, which is responsible for causing amyloidosis and T2D. It is required that the IAPP structure should be well conserved among species, which can help in understanding the mechanistic approaches to be validated, taking into account various animal models and cell culture analysis. The novel phylogenetic and evolutionary studies of IAPP proposed in the paper help in analyzing the significance of effect on IAPP expression further adding analysis to cDNA sequences encoding IAPP precursors from rat, mice, cats, and guinea pigs [64] along with other conidae and primate species.

The underlying mechanisms at gene level can play a crucial role in understanding the interaction of IAPP with cell

**Table 3** hIAPP mRNA sequence identity and alignment with mammalian species

Species sequence alignment	Sequence similarity (%)	E value
<i>Homo sapiens-Mus musculus</i>	76	2e-52
<i>Homo sapiens-Rattus norvegicus</i>	75	4e-49
<i>Homo sapiens-Canis lupus familiaris</i>	74	1e-100
<i>Homo sapiens-Felis catus</i>	77	8e-90
<i>Homo sapiens-Gorilla gorilla</i>	96	0.0
<i>Homo sapiens-Macaca mulatta</i>	98	1e-94
<i>Homo sapiens-Pan troglodytes</i>	99	0.0
<i>Homo sapiens-Sus scrofa</i>	79	2e-97



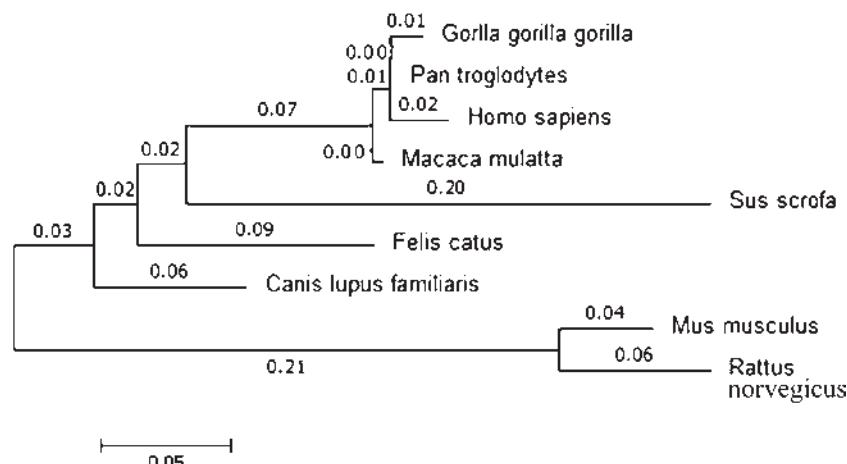
**Fig. 2** Alignment of hpIAPP with eight mammalian species. Legends: (n1) *Homo sapiens*, (n2) *Mus musculus*, (n3) *Rattus norvegicus*, (n4) *Canis lupus familiaris*, (n5) *Felis catus*, (n6) *Gorilla gorilla gorilla*, (n7) *Macaca mulatta*, (n8) *Pan troglodytes*, (n9) *Sus scrofa* (\*sequence conservation)

membranes. This can only be achieved if proper regulatory pathways are studied in in vitro cell culture model or by developing a model organism. Lack of similarity among the upstream promoter, which regulates the IAPP gene expression through various TFs, is seen to be absent in rodents and conidae group. This fact is further confirmed by the evidence that human and other primate species are prone to develop IAPP fibril deposition and T2D. Also, the mRNA sequence is found to be dissimilar, signifying that the TF motifs are not conserved. Another significance is that the mRNA sequences are not transcribed at the same level. It can be inferred that certain features and regulatory mechanisms of IAPP gene are not common among all species and further add to the fact that these animal models may not show significant response to IAPP deposition studies.

All well-assembled nine mammalian sequences have shown that the upstream region of IAPP region is not evolutionary conserved, but faster and a higher rate of substitution among genetic DNA of species has taken place over millions of years. Therefore, the phylogenetic analysis clearly states that the evolution of IAPP gene among nine mammalian species has not been constant over time. Since exon 1 of IAPP gene is non-coding, deleterious mutations in the non-functional region tend to accumulate more mutations overtime

than regulatory regions. This paves the possibility that most of the IAPP SNPs are tolerated and, till date, have been found to be insignificant in various populations. Since human-rodent genomic comparisons of TFBS have been found to be functional in implications of many diseases, a true animal model for IAPP still needs to be established. In this case, it is observed that the human-mouse model for IAPP proves to be weak, as human-rodents are sharing any conservation in the upstream promoter element, and rodents lack regulatory motif which might express IAPP levels metabolically [65]. To explore a possibility of post-translational modification among other mammalian species, MSA of the proIAPP protein is carried out. However, similar results are obtained, which state that the mRNA and hpIAPP sequences are conserved only among the primate group species. However, the rest of the organisms show species divergence.

An important goal of understanding disease initiation is to elucidate how gene regulatory networks evolve and how its evolution causes phenotypic changes in speciation. The next step is to understand the evolution of promoter regulation and their interaction with *cis*-regulatory elements, since proteins folding and misfolding largely depend on gene expression [66]. This genomic landscape of the promoter region and the binding site of various TF can also cause misregulation of



**Fig. 3** The tree is drawn to scale (sum of branch length = 3.75634133 in same units as those of the evolutionary distances used to infer the phylogenetic tree). The analysis involved nine amino acid sequences.

All positions containing gaps and missing data were eliminated. There were a total of 89 positions in the final dataset

**Table 4** Test of molecular clock using the maximum likelihood method

	ln(L)	Parameters	(+G)	(+I)
With clock	−932.524	8	n/a	n/a
Without clock	−733.232	15	n/a	n/a

these programs leading to disease progression, in this case, T2D. As the disease progresses, due to manipulated genome of T2D patients, the effected pancreatic metabolic pathways can be studied clinically. The study and characterization of the complex regulatory mechanism of IAPP at a gene level are tedious tasks. Since a more comprehensive knowledge is required to study the gene regulatory networks, identification of TFBS for IAPP is an important long-term goal to understand its complexity and the initiation of T2D due to protein misfolding. Hence, in this study, TFBSs are established for the first time. Also, it is established that the many TFs are lying in the vicinity of the transcription control sites, and it is likely that they might be playing a crucial role in controlling expression levels of hIAPP. MSA of the primate groups further confirmed the genome-wide set of high levels of conservation in relation to human proximal promoter of IAPP gene. This evolutionary conservation might give an insight that these motifs may be functional. In this study, maximum hits were found to be for the Oct1 TF. This TF shows maximum motifs on both forward and reverse strands, which is further supported by the fact that these POU domain homeoprotein levels are glucose-induced in pancreatic  $\beta$ -cells [67]. GATA1, on the other hand, shows hits pertaining to the fact that insulin-like growth factors have GATA motif in order to initiate their transcriptional activities. In relation with diabetes, pancreatic cell expressions, and insulin differentiating/producing cells, the TFs identified on the hIAPP upstream promoter region are all in relation with the metabolic pathways operating in the pancreas. However, critical analysis of the upstream

promoter region of the IAPP still remains enigmatic. Through the TFBS identification, it is clear that the IAPP upstream promoter region holds key to identifying regulatory factors, which can underlie in identification of mutant IAPP of fibril formation in T2D. This study, therefore, explored the novel instances of variability of IAPP gene in various mammalian species as well as constructed a model and identified motifs that represented the set of binding sites for the promoter elements present, pertaining to genetic regulatory networks of IAPP.

The phylogenetic analysis further supports that the IAPP fibril formation is found specifically in species prone to diabetes, especially in the primate groups. Although, in many cases, rodent species have been useful in identifying disease-specific genes which are also found in humans, but the evolution of certain mammalian genes from lower chordates has observed species divergence. Although mice do not suffer from T2D, transgenic mice models that have made to express hIAPP develop fibrillar deposits and show T2D symptoms [68]. However, this does not eliminate the fact that rodent models for IAPP studies cannot be developed. The use of rodent models may not be helpful to study mutant IAPP fibril formation and their reaction with cell membranes, but new models may be developed if regulatory mechanisms are studied and mutants are generated at the TFBS disrupting IAPP gene regulation, which may further lead to IAPP fibril formation and exhibit signs of T2D. The specification of gene promoter of the rodent species as compared to *H. sapiens* should be kept in mind. Since primate species are the most closely related species to humans, using them as animal models becomes difficult. Although less sequence divergence of regulatory sites, without any doubt, works best for an animal model as compared to more sequence diverged species, animal models can be genetically manipulated, i.e., using gene knock-in and CRISPR/CAS9 techniques [69]. Since no information regarding species divergence of IAPP is known, this study is specifically targeting only the promoter region

**Table 5** Estimates of evolutionary divergence rate between proIAPP sequences among mammalian species

	<i>Homo sapiens</i>	<i>Mus musculus</i>	<i>Rattus norvegicus</i>	<i>Cannis lupus</i>	<i>Felis catus</i>	<i>Gorilla gorilla</i>	<i>Macaca mulatta</i>	<i>Pan troglodytes</i>	<i>Sus scrofa</i>
<i>Homo sapiens</i>									
<i>Mus musculus</i>	0.411								
<i>Rattus norvegicus</i>	0.428	0.094							
<i>Cannis lupus</i>	0.198	0.330	0.362						
<i>Felis catus</i>	0.212	0.394	0.411	0.158					
<i>Gorilla gorilla</i>	0.034	0.378	0.411	0.185	0.198				
<i>Macaca mulatta</i>	0.034	0.394	0.394	0.185	0.198	0.023			
<i>Pan troglodytes</i>	0.023	0.378	0.394	0.171	0.185	0.011	0.011		
<i>Sus scrofa</i>	0.299	0.537	0.577	0.284	0.299	0.299	0.269	0.284	

**Table 6** Analysis of important regulatory TFs having probable functional binding sites on the hAPP upstream promoter region

Transcription factor	Position on IAPP promoter and strandness forward (+) reverse (-)	Position on IAPP promoter and strandness reverse (-)	No. of sites present upstream of the promoter region	Similarity score between registered sequence and the input sequence (0.0–1.0)	Function in relation to pancreatic beta cells Ref.
Oct1	10, 52, 54, 84, 86, 161, 168, 185, 226, 359, 405, 434, 443, 458, 462, 463, 465, 467, 468, 576, 588, 713, 742, 745, 748, 770, 811, 813, 815, 816, 818, 832, 838, 928, 965, 974, 990, 993, 1011, 1012, 1021, 1058, 1076, 1078, 1079, 1083, 1133, 1215, 1217, 1226, 1406, 1425, 1447, 1449, 1451, 1475, 1506, 1523, 1555, 1588, 1608, 1617, 1646, 1647, 1654, 1657, 1681, 1682, 1684, 1687, 1693, 1707, 1793, 1799, 1804, 1872, 1919, 1932, 1938, 1940, 1943, 1997, 2005, 2007, 2010, 2038, 2059, 2069, 2093, 2096, 2106, 2124, 2127, 2130, 2154, 2176, 2178, 2180, 2181, 2204, 2284, 2342, 2356, 2357, 2365, 2378, 2384, 2408, 2420, 2476, 2482, 2492, 2530, 2540, 2588, 2589, 2594, 2637, 2671, 2690, 2708	60, 64, 89, 93, 158, 219, 227, 292, 365, 430, 434, 440, 445, 468, 628, 654, 740, 751, 761, 766, 783, 789, 805, 816, 834, 837, 839, 843, 913, 917, 931, 965, 966, 980, 993, 993, 1008, 1013, 1023, 1027, 1033, 1045, 1057, 1071, 1201, 1219, 1225, 1248, 1289, 1356, 1444, 1452, 1452, 1553, 1556, 1593, 1610, 1632, 1648, 1648, 1654, 1667, 1688, 1715, 1727, 1754, 1772, 1791, 1827, 1845, 1849, 1911, 1929, 1931, 1933, 1935, 1937, 1939, 1943, 1943, 1960, 1998, 2007, 2035, 2103, 2109, 2125, 2165, 2281, 2362, 2370, 2376, 2385, 2409, 2436, 2454, 2477, 2481, 2483, 2483, 2489, 2525, 2533, 2539, 2637, 2638, 2642, 2644, 2669, 2687	231	0.91	Role in insulin gene expression and regulation of pancreatic β-cells [36, 37]
GATA1	81, 133, 150, 166, 173, 351, 396, 411, 471, 472, 473, 481, 723, 775, 1061, 1063, 1096, 1098, 1113, 1152, 1154, 1243, 1283, 1328, 1501, 1520, 1641, 1809, 1877, 1904, 1924, 2033, 2116, 2180, 2216, 2227, 2245, 2256, 2258, 2294, 2296, 2348, 2349, 2350, 2500, 2610, 2676, 2689	28, 28, 33, 218, 252, 261, 366, 459, 528, 557, 645, 645, 645, 734, 736, 755, 757, 792, 794, 829, 867, 880, 890, 1154, 1200, 1206, 1288, 1328, 1373, 1457, 1536, 1629, 1631, 1712, 1714, 1733, 1765, 1777, 1784, 1818, 1829, 1844, 1846, 1846, 1848, 1956, 1968, 1972, 1974, 2070, 2139, 2168, 2312, 2331, 2333, 2532, 2573, 2668	120	0.85	Transcriptional binding site present on insulin promoter region [38]
CAP	5, 11, 26, 150, 156, 197, 223, 332, 357, 366, 423, 441, 445, 457, 473, 530, 538, 556, 600, 660, 715, 721, 735, 756, 762, 767, 799, 895, 971, 998, 1000, 1006, 1129, 1132, 1144, 1159, 1183, 1293, 1335, 1348, 1375, 1462, 1507, 1594, 1665, 1680, 1688, 1714, 1760, 1789, 1794, 1837, 1883, 1896, 1948, 1950, 1979, 2018, 2046, 2048, 2068, 2141, 2223, 2338, 2344, 2428, 2500, 2578, 2683	—	69	0.8	Plays crucial role in lactose metabolism. Activates cAMP levels when glucose level rises [39]
AP4	241, 331, 353, 356, 379, 382, 419, 422, 529, 623, 1128, 1143, 1158, 1262, 1788, 1978, 2196, 2208, 2224, 2253, 2429, 2630	241, 2478, 347, 356, 422, 623, 1322, 1484, 1493, 1521, 1639, 2049, 2052, 2115, 2143, 2149, 2194, 2208, 2215, 2224, 2346, 2355	68	0.89	Transcription factors expressed in differentially induced genes in RASGrf1 KO pancreatic islets and regulatory networks in insulin-producing cells' differentiation [40, 41]
GATA2	150, 166, 173, 473, 481, 536, 593, 713, 852, 981, 1063, 1098, 1154, 1193, 1243, 1328, 1712,	33, 218, 400, 528, 557, 736, 757, 794, 880, 1122, 1154, 1200, 1206, 1256, 1288, 1328, 1373,	59	0.78	[42]

**Table 6** (continued)

Transcription factor	Position on IAPP promoter and strandness forward (+) reverse (-)	Position on IAPP promoter and strandness reverse (-)	No. of sites present upstream of the promoter region	Similarity score between registered sequence and the input sequence (0.0–1.0)	Function in relation to pancreatic beta cells Ref.
S8	1877, 1924, 2033, 2116, 2180, 2216, 2296, 2350, 2500, 2610	1457, 1536, 1592, 1631, 1714, 1733, 1777, 1848, 1938, 1974, 2139, 2333, 2532, 2573, 2668	43	0.73	Helps in differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells [43]
SP1	66, 82, 89, 182, 729, 836, 935, 1046, 1053, 1222, 1688, 1998, 2065, 2128, 2182, 2261, 2405, 2409, 2590, 2648	68, 84, 159, 726, 830, 838, 937, 1009, 1019, 1039, 1073, 1224, 1523, 1552, 1602, 1690, 2122, 2263, 2515, 2542, 2592, 2634, 2650	41	0.89	Transcriptional elements present in insulin gene responding to glucose Sp1 motif present on insulin promoter genes [44]
MEF2	258, 260, 283, 308, 344, 346, 394, 430, 480, 553, 567, 607, 608, 609, 991, 1096, 1101, 1103	197, 265, 676, 679, 680, 681, 699, 700, 702, 1127, 1181, 1185, 1201, 1205, 1248, 1332, 1734, 1738, 1780, 1821, 1881, 2139	38	0.91	Controls pancreatic $\beta$ and $\delta$ -cell mass through HDACs [45]
SOX5	53, 950, 1002, 1023, 1045, 1050, 1063, 1210, 1218, 1411, 1441, 1547, 1637, 2165, 2604, 2613, 2622, 2634, 2635	12, 13, 182, 442, 834, 952, 970, 1047, 1440, 1812, 2167, 2330, 2388, 2405, 2407, 2409,	38	0.90	Expression in developing pancreas [46]
vMYB	45, 75, 113, 578, 838, 860, 902, 937, 1009, 1217, 1430, 1552, 1574, 1645, 1731, 2130, 2368, 2382, 2484, 2557	11, 98, 286, 483, 570, 723, 823, 905, 948, 1556, 1653, 1696, 1794, 1954, 2354, 2446, 2502, 2691, 2694	2691, 2694	0.71	Transcriptional stimulation of insulin gene [47]
CEBPA	107, 231, 272, 379, 424, 477, 502, 517, 558, 580, 587, 744, 1554, 1636, 1798, 2073, 2565, 2658	28, 634, 1042, 1319, 1337, 1540, 1758, 1759, 1843, 1844, 1855, 1886, 1896, 1978, 2070,	37	0.76	Involved in pancreatic fate of human embryonic stem cells [48]
NF1	1185, 1350, 1567, 2118, 2123, 2266, 2553	55, 522, 788, 823, 834, 908, 983, 1052, 1228, 1545, 1556, 2267, 2354, 2482, 2502, 2519, 2542, 2645, 2698	36	0.83	Regulates ALAS gene for insulin repression along with HNF3 [49]
ROR	21, 281	1875	32	0.87	—
TST1	RORA 1: 49, 79, 188, 296, 302, 521, 1585, 1887, RORA 2: 79, 649, 841, 2644, 3, 70, 495, 791, 814, 1431, 2039, 2246, 2479, 2632, 2636, 2651	RORA 1: 180, 541, 638, 720, 943, 1188, 1788, 1803, 1824, 2360, 2586 RORA 2: 452, 638, 648, 720, 943, 1188, 2160, 332, 441, 570, 711, 1013, 1117, 1251, 1462, 1655, 1771, 1794, 1805, 1827, 2007, 2348, 2404, 2589, 2638	30	0.85	Activates somatostatin receptor 1 gene expression in pancreatic beta cells [50]
SRF	47, 251, 658, 1231, 1233, 1234, 1842, 1845, 1936, 1938, 1939, 2029, 2030, 2271, 2371	44, 657, 720, 1231, 1233, 1842, 1844, 1852, 1936, 1938, 2029, 2168, 2300, 2388, 2455	30	0.84	Regulates insulin gene and enriched expression in pancreatic $\beta$ -cells [51]
STAT	746, 808, 1126, 1252, 1726, 1987, 2007, 2125, 2397, 2545, 2589, 2618	40, 58, 576, 746, 808, 955, 1050, 1317, 1415, 1437, 1527, 1987, 2007, 2397, 2418, 2457	28	0.95	Linked to diabetes pathogenesis [52]
CDXA	368, 449, 488, 1054, 1566, 1820, 1831, 2132, 2190, 2262, 2395, 2640	132, 812, 912, 921, 1053, 1075, 1082, 1480, 1508, 1776, 1798, 2030, 2491, 2579, 2593, 2651	28	0.88	Marks distinct endoderm territories toward pancreatic fate map [53]
E2F	22, 231, 524, 542, 637, 1211, 1363, 1437, 2181, 2512, 2535	5, 19, 250, 312, 498, 545, 1296, 1328, 1357, 1365, 1404, 1818, 2399, 2537	25	0.79	Controls cell cycle regulation of pancreatic cells [54]
E4BP4	15, 56, 185, 617, 1671, 2124, 2216, 2245, 2597	69, 353, 744, 835, 909, 959, 1030, 1221, 1503, 1671, 1756, 1846, 2245, 2483, 2597, 2646	25	0.77	—

**Table 6** (continued)

Transcription factor	Position on IAPP promoter forward (+) reverse (−)	Position on IAPP promoter and strandness	No. of sites present upstream of the promoter region	Similarity score between registered sequence and the input sequence (0.0–1.0)	Function in relation to pancreatic beta cells Ref.
HNF1	178, 934, 1045, 1049, 1456, 1524, 2105, 2123, 2185, 2379	64, 145, 151, 673, 826, 834, 933, 1047, 1657, 1901, 2126, 2259, 2378	23	0.87	A clock-controlled output gene inhibits ARNT promoter activity in mouse pancreatic islets [56]
ARP1	25, 356, 683, 1107, 1334, 1347, 1769, 1883, 1979, 2003	276, 300, 958, 1164, 1494, 1893, 2192, 2218, 2236, 2465, 2670, 2678	22	0.86	Mutations in hepatocyte nuclear factor 1α ( <i>HNF-1α</i> ) lead to maturity-onset diabetes of the young type 3 as a result of impaired insulin secretory response in pancreatic β-cells. – [57]
HOX13	113, 745, 1472, 1542, 1939, 2100, 2531	66, 821, 936, 1043, 1064, 1081, 1543, 1565, 1652, 2105, 2254, 2262, 2401, 2551	21	0.79	Plays a major role in pancreatic development [58]
NF-κB	179, 495, 620, 1015, 1198, 1199, 1286, 2010, 2162, 2163	243, 494, 800, 1197, 1198, 1285, 1286, 2010, 2011, 2038, 2383	21	0.80	Involved in progression and metastasis of human pancreatic cancer along with AP1 DNA-binding transcription factor [59]
E2	45, 75, 197, 249, 1232, 1310, 1533, 2068, 2369	45, 75, 197, 721, 1310, 1533, 1948	16	0.81	Network formation and gene activation signals mediating insulin transcription. E2 binding site required for transcription of the insulin gene [60, 61]
MYB	161, 233, 517, 587, 1600, 2073	311, 432, 863, 1978, 2265	11	0.86	Linked to pancreatic cancer [62]

and its conservation. It further elucidates regulatory transcription network based on new sequences targeted by never-before-reported TFs, which might be functionally regulating IAPP gene transcription.

In conclusion, the transcriptional analysis of IAPP gene can identify essential mutants that may form amyloid fibrils. Amyloid fibrils display high content of cross- $\beta$ -sheet structures regardless of their sequences and native structures. The potential state of mutant hIAPP conformational states could be further tested both by *in vitro* and by *in silico* through thermodynamics methods. This study can initiate basic working of IAPP gene and metabolic regulation in IAPP-induced T2D.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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REVIEW ARTICLE

# The impact of diabetes on tuberculosis treatment outcomes: evidence based on a cumulative meta-analysis

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**Abstract** Diabetes mellitus (DM) is a known risk factor for tuberculosis (TB). It is also associated with worse tuberculosis treatment outcomes. This study aimed to perform a systematic review and meta-analysis to quantitatively summarize evidence for the impact of diabetes on tuberculosis outcomes. We searched PubMed, Cochrane, and Web of Science from January 1980 to July 2015. The combinations of key words used were (((("Tuberculosis"[Mesh]) OR (Tuberculosis OR pulmonary tuberculosis OR TB))) AND ((“Diabetes

Mellitus”[Mesh]) OR (Diabetes Mellitus OR Diabetes OR DM))) AND (Outcome OR sequelae OR treatment OR therapy OR culture conversion OR failure OR death OR relapse OR recurrence)). We reviewed the full text of 70 papers and included 54 studies of which 16 reported culture conversion at 2 to 3 months, 24 reported the combined outcome of failure and death, 34 reported death, 8 reported relapse, and 7 reported drug-resistant recurrent tuberculosis. Patients with diabetes have an odds ratio (OR) through this cumulative meta-analysis for the combined outcome of failure and death of 1.96 (95 % CI, 1.64 to 2.33). The OR of death during tuberculosis treatment among the 34 unadjusted studies is 1.83 (95 % CI, 1.61 to 2.07). Diabetes is also associated with an increased risk of relapse (OR, 1.97; 95 % CI, 1.42 to 2.74). The OR for studies assessing sputum culture conversion after 2 to 3 months of tuberculosis therapy is 1.71 (95 % CI, 1.50 to 1.94). Diabetes increases the risk of failure and death combined, death, and relapse among patients with tuberculosis. This study highlights a need for increased attention to treatment of tuberculosis in people with diabetes, which may include testing for suspected diabetes, improving glucose control, and increasing clinical and therapeutic monitoring.

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**Keywords** Diabetes · Tuberculosis · Treatment outcomes · Death · Relapse

## Introduction

Tuberculosis (TB) is an infectious disease caused by the intracellular bacterial pathogen, *Mycobacterium tuberculosis* [1].

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In 2014, there were an estimated 11 million people living with TB, 9.6 million new cases and 1.5 million deaths (WHO 2014).

Despite the availability of effective therapy, TB continues to infect an estimated one third of the world's population, to cause disease in 8.8 million people per year, and to kill 1.6 million of those afflicted [2]. In recent years, strong evidence has been gathered to confirm a link between TB and yet another disease: diabetes mellitus [3].

The number of people with diabetes is expected to rise to at least 592 million by 2035 [4]. The alarming rise in cases of type 2 diabetes poses a serious threat to global tuberculosis control. People with diabetes are three times more likely to develop active tuberculosis than those without diabetes, and there are now more tuberculosis patients with concomitant diabetes than with HIV infections [5].

In addition, studies that screened for DM among TB patients reported a wide range of DM prevalence among TB patients, ranging from 1.9 % to as high as 35 % [6, 7]. There is a close link between diabetes and tuberculosis, but not a systematic analysis to both clarify and quantify the association between DM and TB outcomes, including persistence of sputum culture positivity, failure, death, and relapse. We conducted a systematic review and meta-analysis to make further clarification and quantification of the association between DM and these outcomes, and this will inform public health measures.

## Methods

### Data sources and searches

We searched PubMed, EMBASE, and Web of Science from January 1980 to July 2015 for studies of the association between DM and TB treatment outcomes (Table 1).

**Table 1** Eligible studies involved at least one factor related to outcome, sequelae, treatment, therapy, culture conversion, failure, death, relapse OR recurrence

Search terms	
1	Tuberculosis[Mesh]
2	Tuberculosis OR pulmonary tuberculosis OR TB
3	1 OR 2
4	Diabetes Mellitus[Mesh]
5	Diabetes Mellitus OR Diabetes OR DM
6	4 OR 5
7	Outcome OR sequelae OR treatment OR therapy OR culture conversion OR failure OR death OR relapse OR recurrence
8	3 And 6 And 7

### Study selection

Studies were included if they met the following criteria: (1) They were peer-reviewed reports of studies involving human participants receiving pharmacologic anti-mycobacterial treatment for TB disease. (2) They provided or permitted the computation of an effect estimate of the relationship between DM and at least one of the following five TB treatment outcomes: proportion of treated patients who experienced culture conversion at 2 to 3 months, the combined outcome of treatment failure and death, death, relapse, or recurrence with drug-resistant (DR) TB. Treatment failure was defined as sputum smear or culture positivity at 5 months or later during treatment [8, 9]. (3) They defined DM as any of the following:

baseline diagnosis by self-report, medical records, fasting blood glucose (FBG)  $\geq 126$  or  $\geq 140$  mg/dL (to reflect the present and past American Diabetes Association Guidelines and World Health Organization (WHO) recommendations for the diagnosis of DM [10, 11]), non-FBG  $\geq 200$  mg/dL, or treatment with oral hypoglycemic medications or insulin.

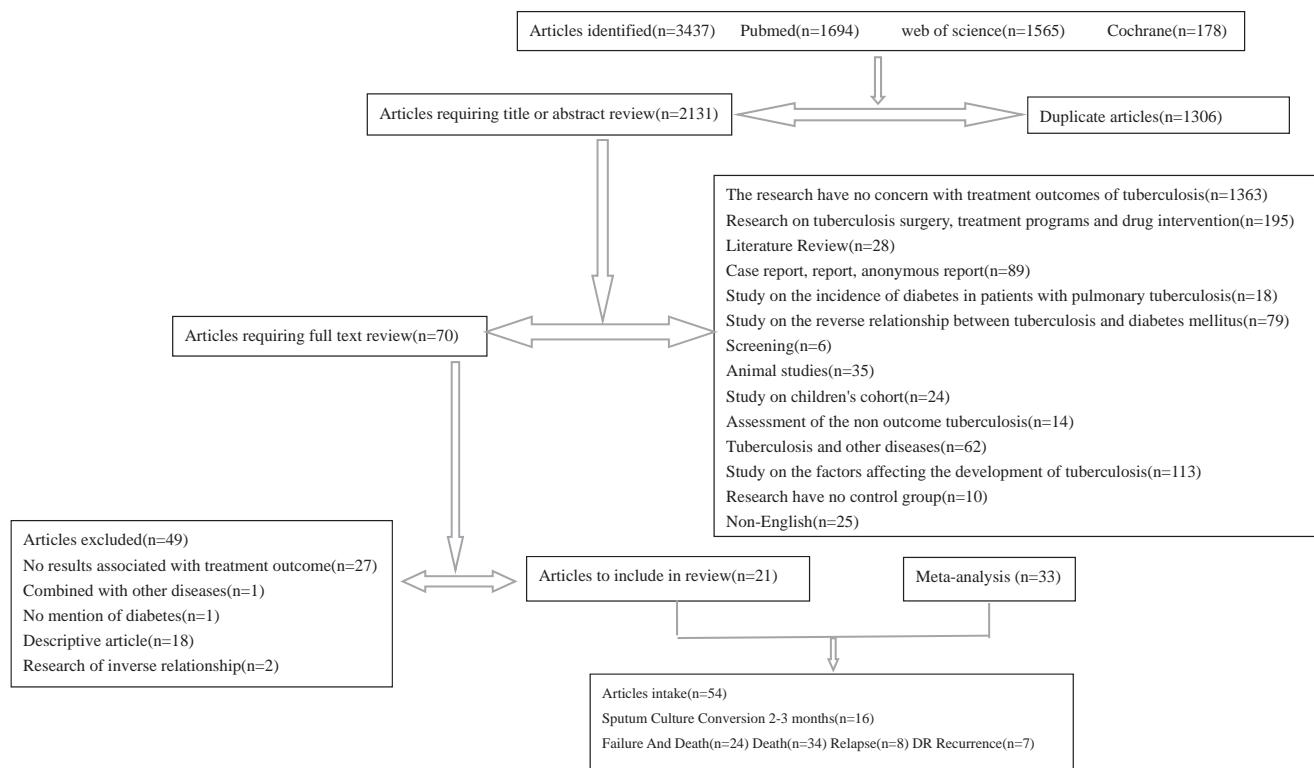
We excluded the following: research that have no concern with treatment of tuberculosis; research on tuberculosis surgery, treatment programs, and drug intervention; literature review; case report, report, and anonymous report; study on the incidence of diabetes in patients with pulmonary tuberculosis; study on the reverse relationship between tuberculosis and diabetes mellitus; screening; animal studies; study on children's cohort; assessment of non-outcome tuberculosis; tuberculosis and other diseases; study on the factors affecting the development of tuberculosis; research have no control group; non-English articles; no results associated with treatment outcome; combined with other diseases; no mention of diabetes; descriptive article; and research of inverse relationship.

### Data extraction and quality assessment

Two reviewers (XH) independently performed study selection according to eligibility criteria. Disagreements were resolved by discussion or by a third reviewer (YZ). Detailed information was extracted from each study including the authors, publication year, country, type of study, type of TB, DM definition, total population, the population with DM, and the outcomes.

## Results

We distinguished and filtrated 2131 papers by titles and abstracts. We excluded 2061 papers: research that have no concern with treatment outcomes of tuberculosis, research

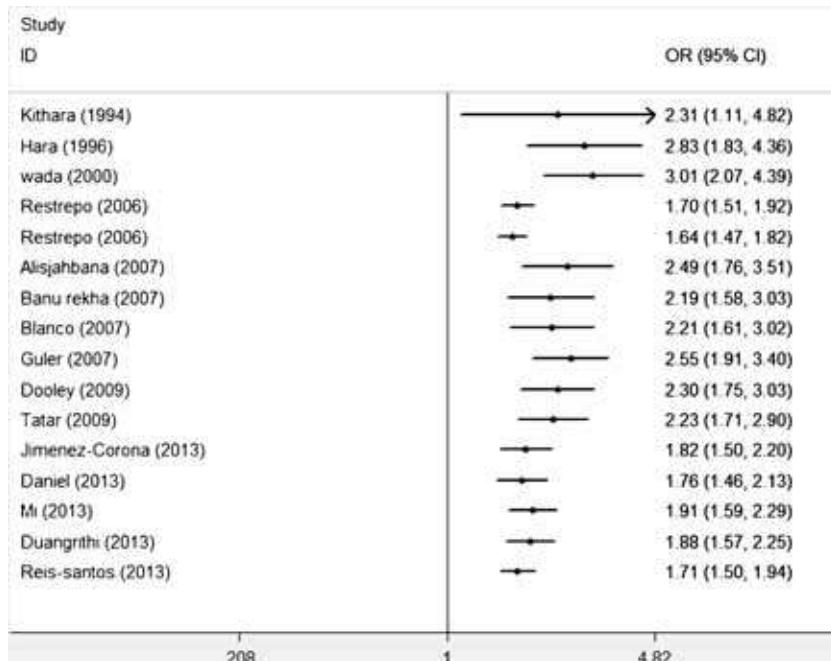


**Fig. 1** Literature search for studies on the association between DM and TB outcomes

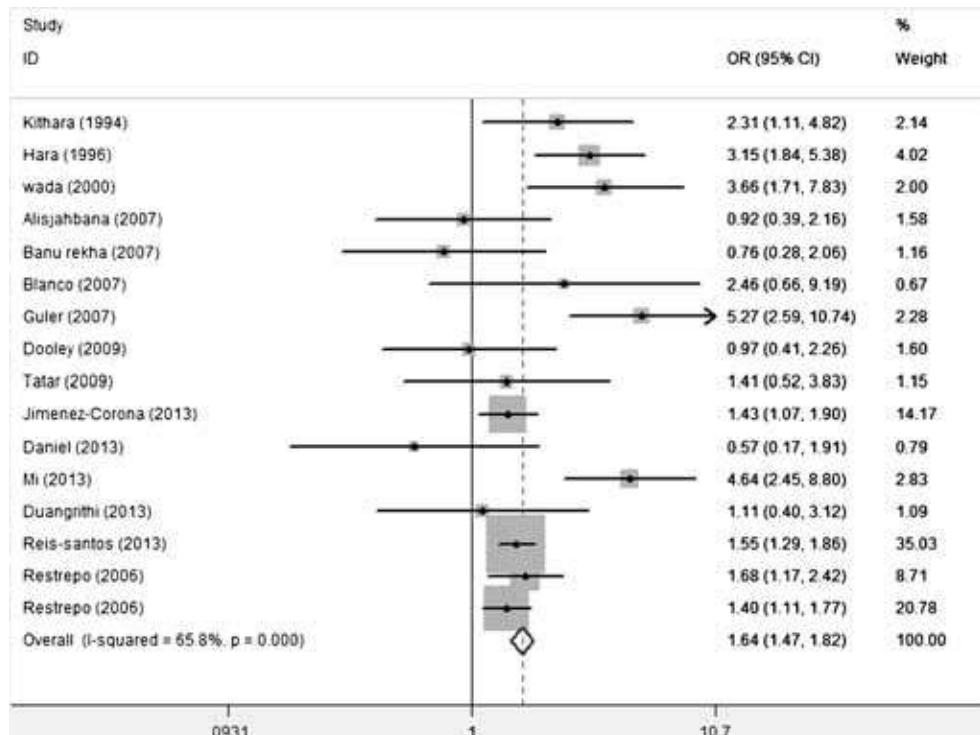
on tuberculosis surgery, treatment programs and drug intervention, literature review, case report (anonymous report), study on the incidence of diabetes in patients with pulmonary tuberculosis, study on the reverse relationship

between tuberculosis and diabetes mellitus, screening, animal studies, study on children's cohort, assessment of non-outcome tuberculosis, tuberculosis and other diseases, study on the factors affecting the development of

**Fig. 2** OR and 95 % CI of the sputum culture positivity at 2 to 3 months on cumulative meta-analysis



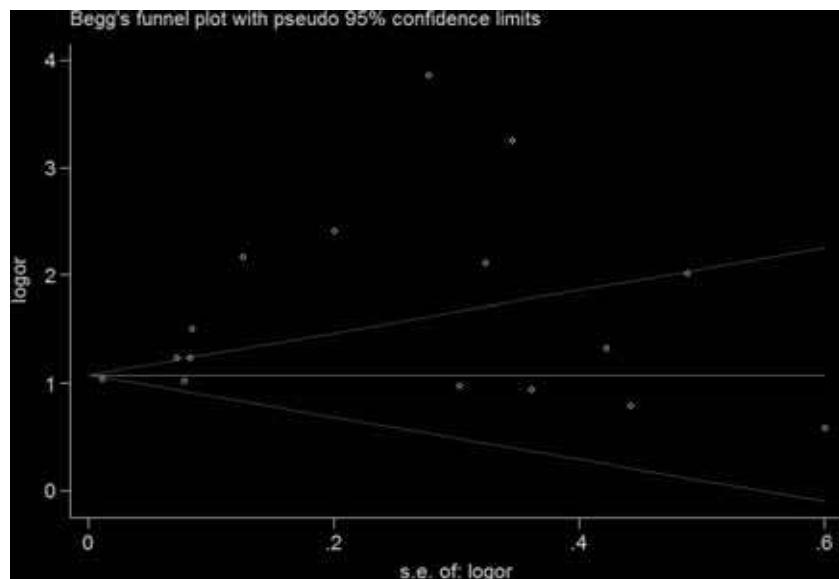
**Fig. 3** OR and 95 % CI of sputum culture positivity at 2 to 3 months



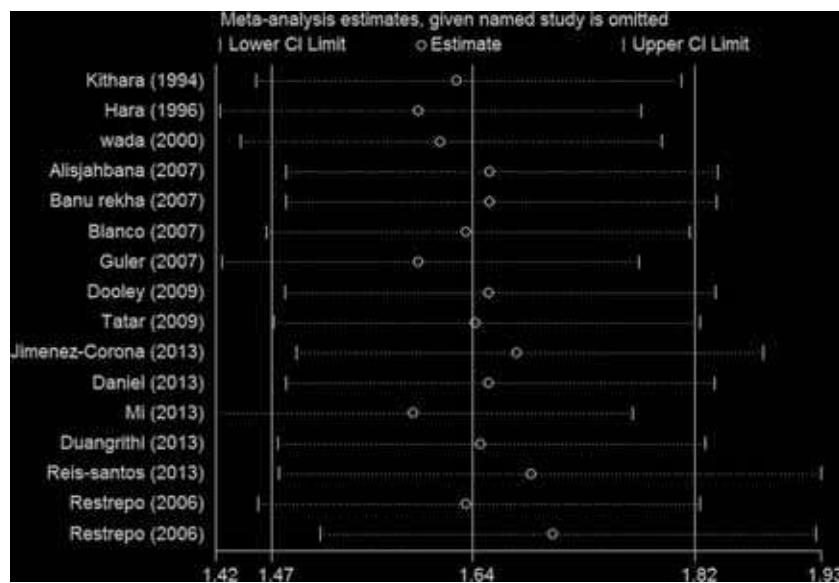
tuberculosis, research that have no control group, and non-English articles (Fig. 1). The full texts of the remaining 70 papers were analyzed, and on the basis of that review, we excluded 49 articles because they did not have the results associated with treatment outcome (27), they combined with other diseases (1), they did not mention diabetes (1), they are descriptive articles (18), and the articles show in inverse

relationship (2). We included 54 studies of which 16 reported culture conversion at 2 to 3 months, 24 reported the combined outcome of failure and death, 34 reported death, 8 reported relapse, and 7 reported recurrence with drug-resistant tuberculosis (Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21). The included articles were written in English (Table 2).

**Fig. 4** Funnel plot for sputum culture positivity at 2 to 3 months



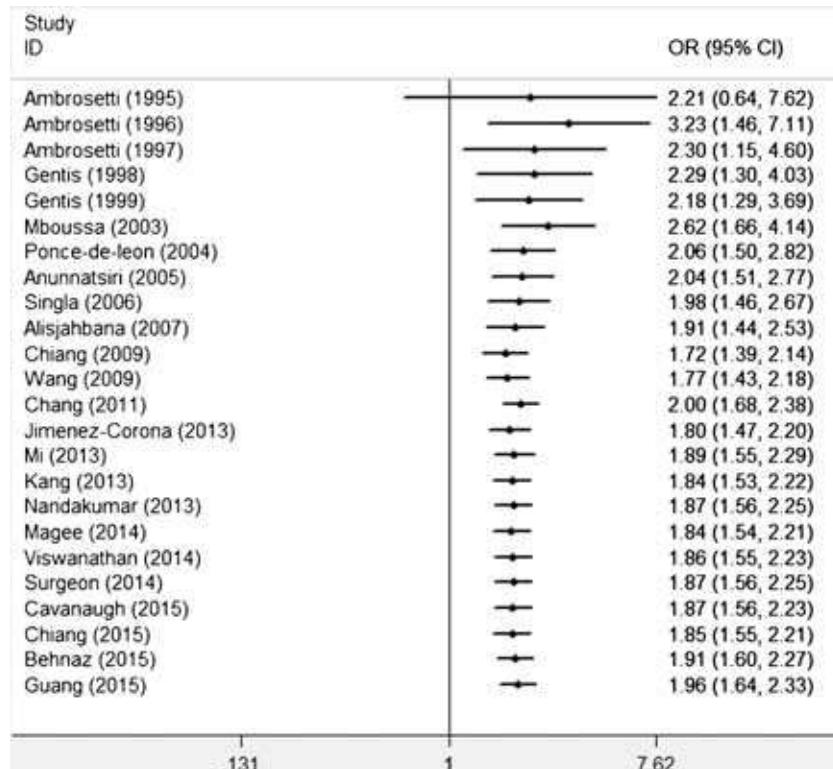
**Fig. 5** Sensitivity analysis for sputum culture positivity at 2 to 3 months



### Sputum culture conversion at 2 to 3 months

The OR of the sputum culture positivity at 2 to 3 months was 1.71 (95 % CI, 1.50 to 1.94). Among the 16 studies that assessed the risk of remaining sputum culture positive after 2 to 3 months, we found statistical heterogeneity is  $I^2 = 65.8\%$ . A publication bias was shown by Begg's rank-correlation method  $P > |z| = 0.685$  (continuity corrected) and the Egger weighted-regression method ( $P = 0.007$ ) (Table 3).

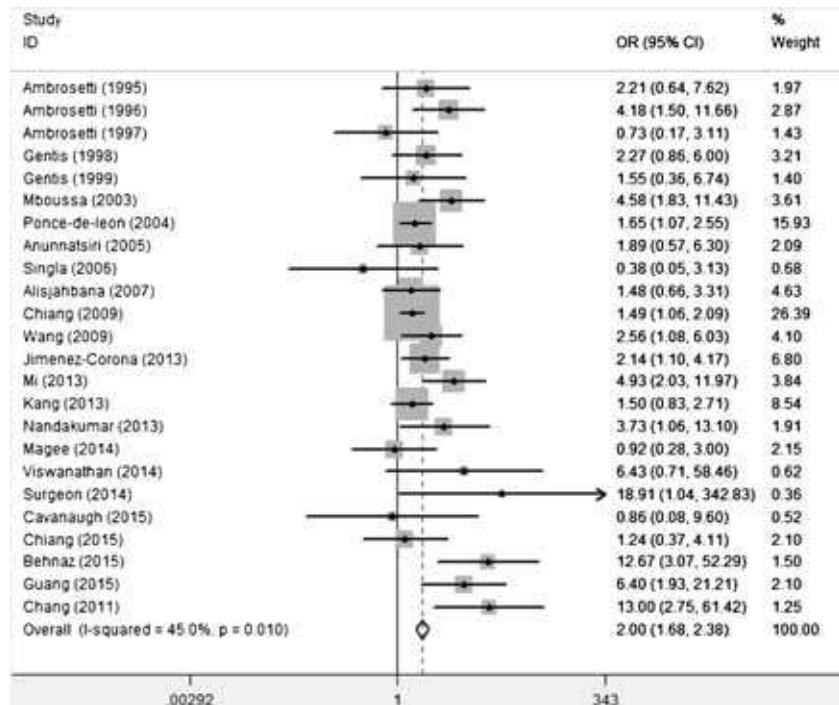
**Fig. 6** OR and 95 % CI of failure and death on cumulative meta-analysis



### Failure and death

The OR of the combined outcome, failure, and death among the 24 studies that included both outcomes was 1.96 (95 % CI, 1.64 to 2.33). We found the statistical heterogeneity is 45 %. Publication bias was shown by Begg's rank-correlation method  $P > |z| = 0.673$  (continuity corrected) and the Egger weighted-regression method  $P > |t| = 0.076$ .

**Fig. 7** OR and 95 % CI of failure and death



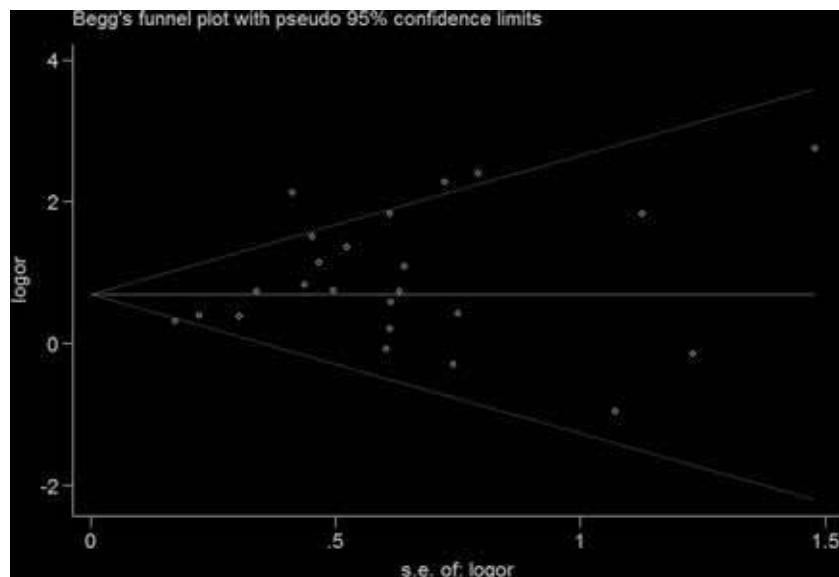
## Death

Among the 34 studies that compared the risk of death during TB treatment in patients with DM versus patients without DM, we found that heterogeneity was 70.2 %. The OR from the effects analysis was 1.83 (95 % CI, 1.61 to 2.07). Although Begg's test suggested publication bias ( $P = 0.001$ ), Egger's test was not significant ( $P = 0.284$ ).

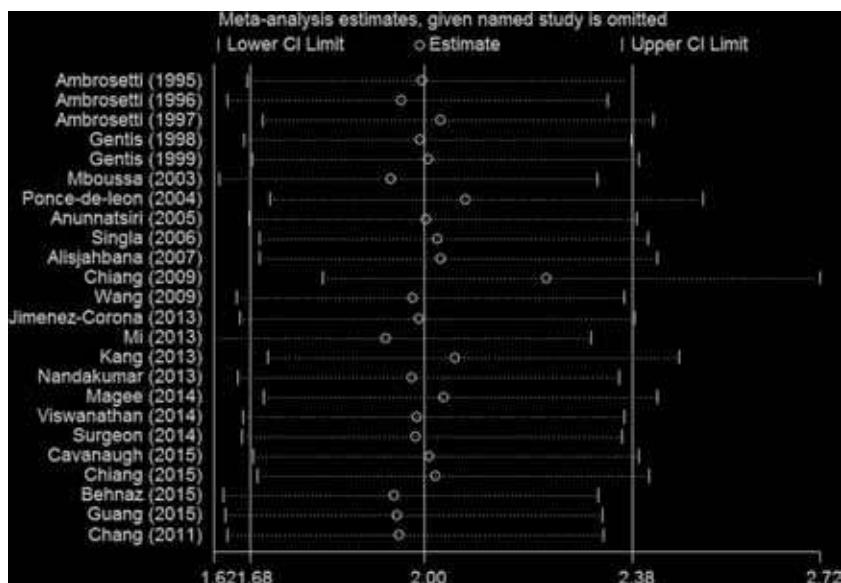
## Relapse

Among the eight studies that assessed the risk of TB relapse, the effects pooled OR was 1.97 (95 % CI, 1.42 to 2.74) for relapse after TB cure or treatment completion among patients with DM versus patients without DM and the heterogeneity was 63.8 %. There is no evidence for publication bias by Begg's test ( $P = 0.108$ ) or Egger's test ( $P = 0.097$ ).

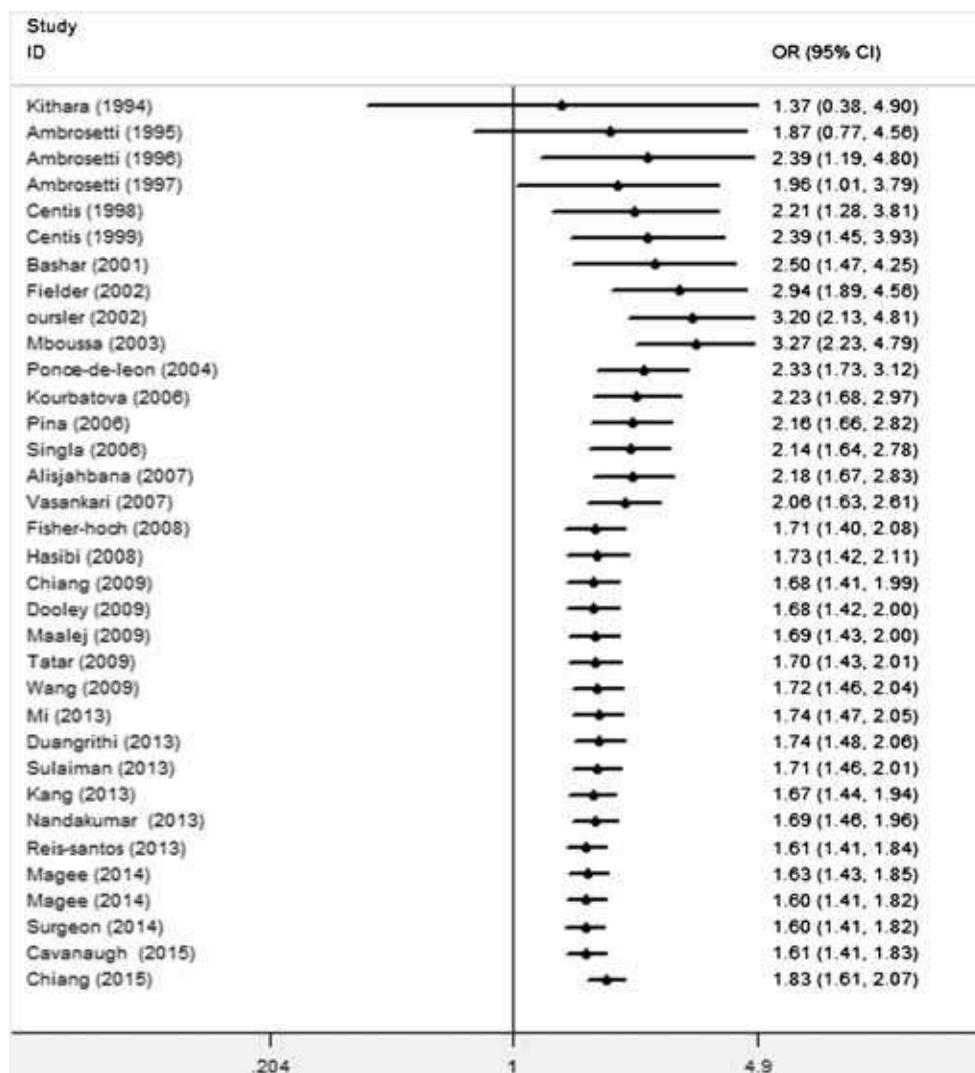
**Fig. 8** Funnel plot for failure and death

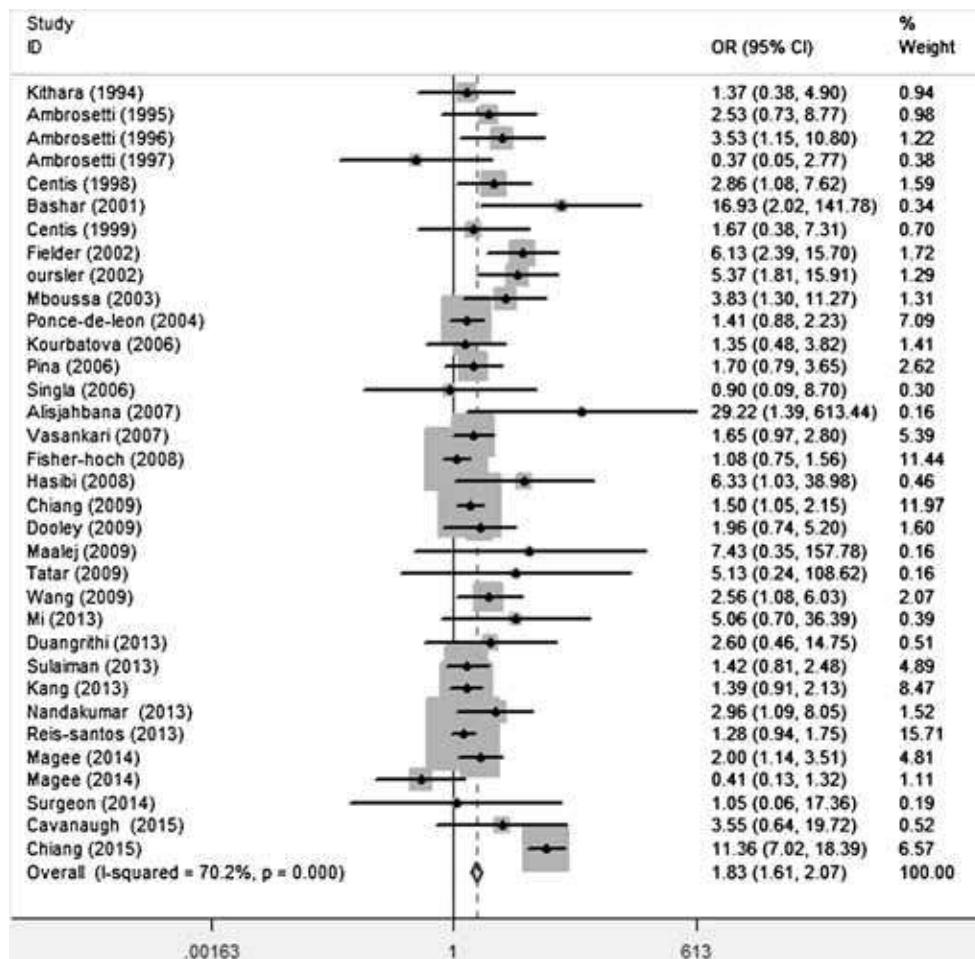


**Fig. 9** Sensitivity analysis for failure and death



**Fig. 10** OR and 95 % CI of death on cumulative meta-analysis

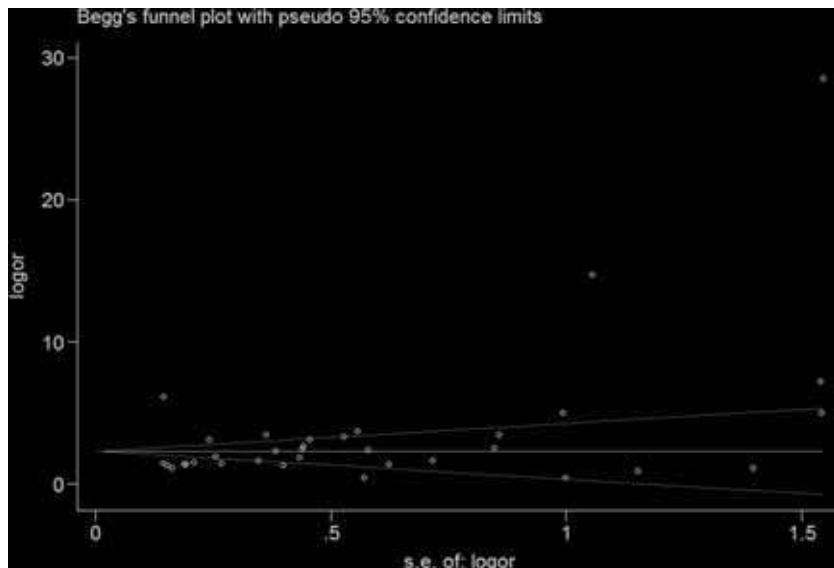


**Fig. 11** OR and 95 % CI of death

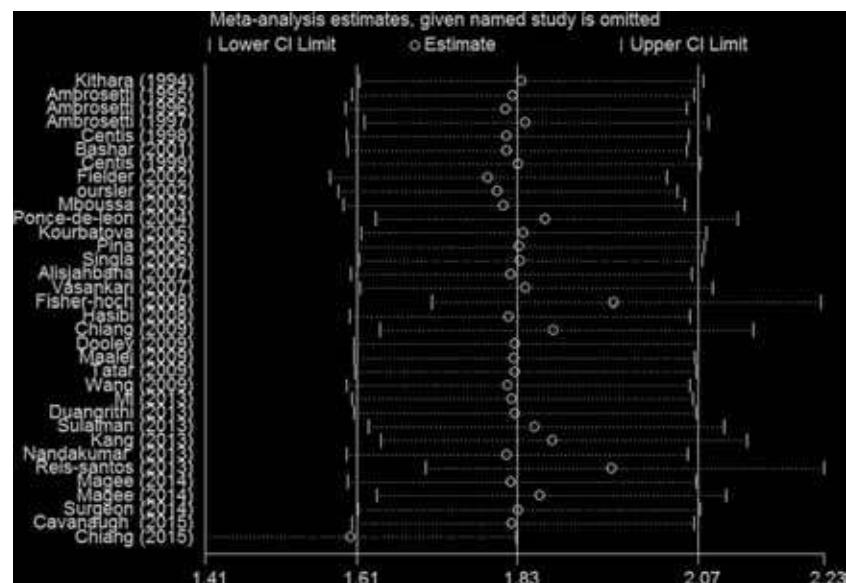
### Drug-resistant recurrent disease

The effects pooled OR was 1.50 (95 % CI, 1.09 to 2.05) for the seven studies that assessed the odds of developing recurrent

TB that is DR. There was no evidence for heterogeneity among the studies that assessed this outcome. We did not find evidence for publication bias by Begg's test ( $P = 0.230$ ) or Egger's test ( $P = 0.504$ ).

**Fig. 12** Funnel plot for death

**Fig. 13** Sensitivity analysis for death



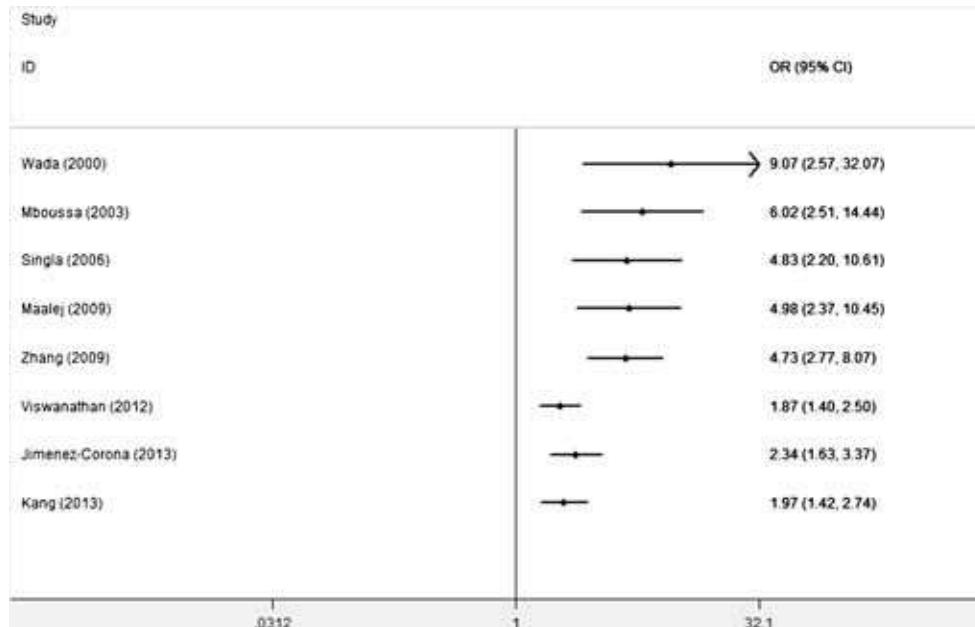
## Discussion

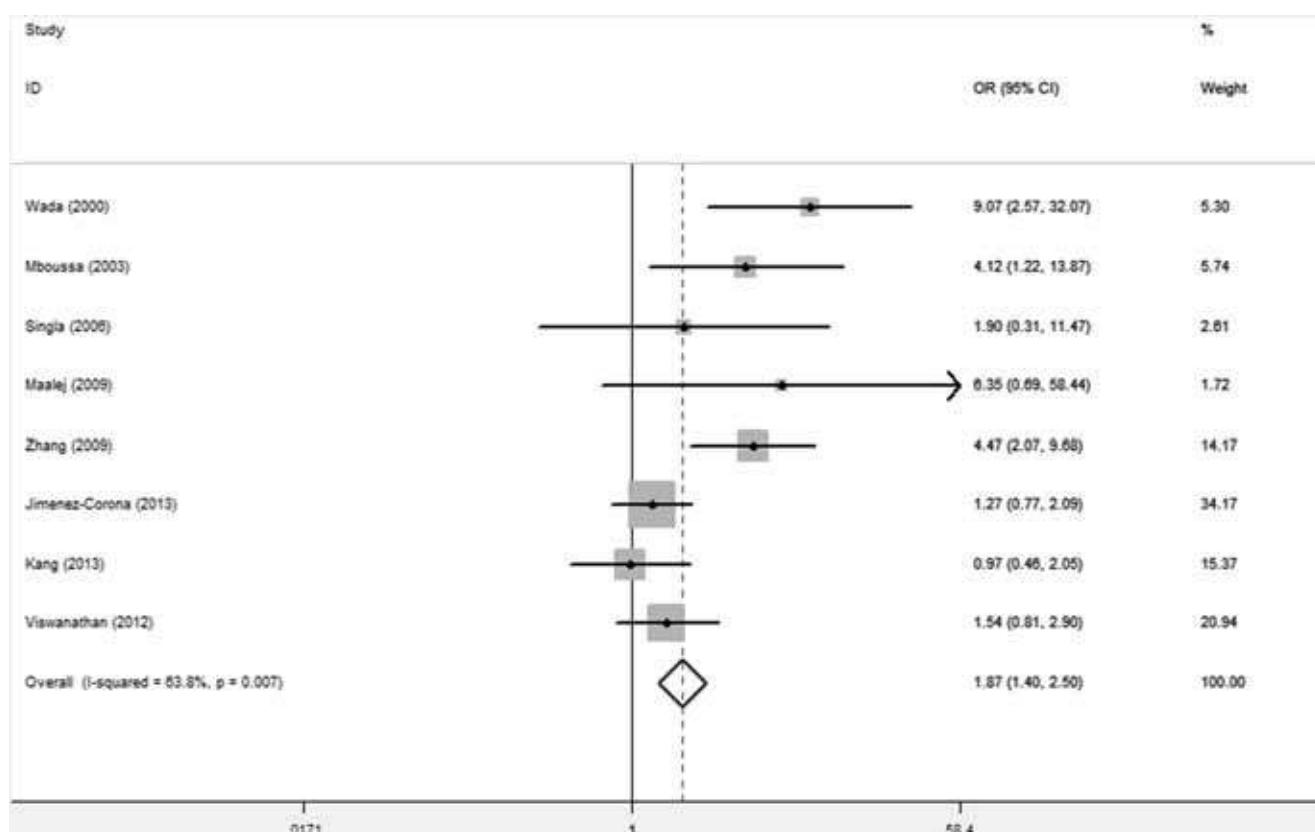
This cumulative meta-analysis of the impact of DM on the treatment outcomes of TB determines that DM increases the risk of the combined outcome of failure and death, death, and relapse.

DM increases susceptibility to a wide variety of infections including pulmonary TB. Existing studies suggest that DM is to increase the risk of active tuberculosis, namely by the latent infections into activity. The sugar metabolism disorder induced by diabetes causes the sugar content of the tissue to be increased, which

is beneficial to the growth and reproduction of the nuclear bacteria. High blood sugar can inhibit the phagocytic ability, well-controlled diabetes whose leukocyte bactericidal capacity was significantly stronger than those with poor control. The bacterial phagocytic capacity, resistance to tubercle bacilli, and T-cell function were shown to be depressed in peritoneal macrophages from mice with experimentally induced diabetes. Hyperglycemia and ketone bodies that accumulate provide a source of nutrition and a suitable acidic environment for tuberculosis; meanwhile, DM microvasculature disease involving the lungs provides a suitable place for

**Fig. 14** OR and 95 % CI of relapse on cumulative meta-analysis

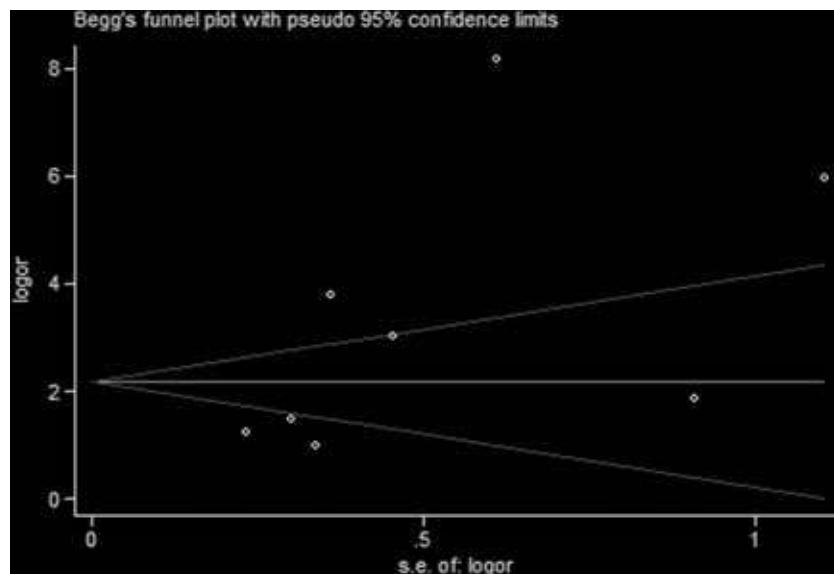


**Fig. 15** OR and 95 % CI of relapse

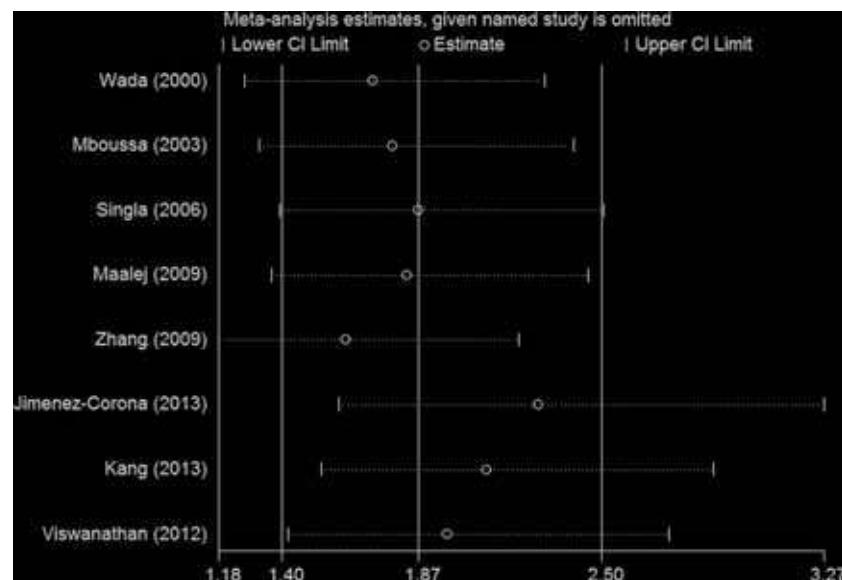
*M. tuberculosis* invasion and breeding. These studies provided strong evidence to determine the relationship between TB and DM.

The OR of the sputum culture positivity at 2 to 3 months in this cumulative meta-analysis tends to be stable.

The publication bias, shown by Begg's rank-correlation method, was not statistically significant, and the Egger weighted-regression method was statistically significant. We performed a sensitivity analysis to determine the influence of individual dataset on the summary OR by consecutively deleting individual studies. The combined OR

**Fig. 16** Funnel plot for relapse

**Fig. 17** Sensitivity analysis for relapse



was not significantly altered by deleting each selected study sequentially, which indicated that our results were stable and robust.

The OR of the combined outcome, failure, and death was roughly the same over the past 5 years. No statistically significant publication bias was shown by Begg's rank-correlation method and Egger weighted-regression method. The study of Chiang [23] has a large sample size and a big weight of 26.39 %. So statistical heterogeneity is 45 % with this study and 38 % without it.

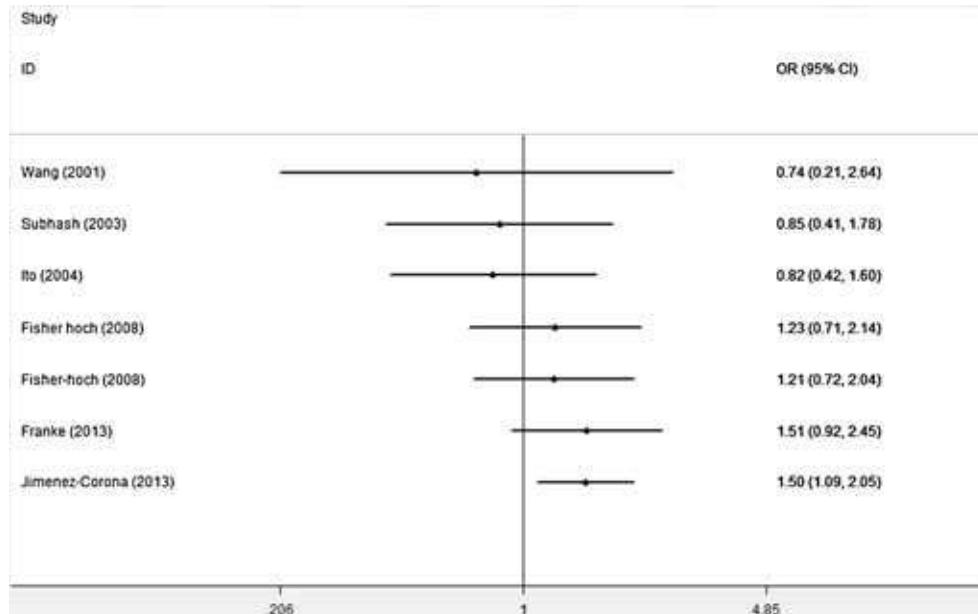
Among the 34 studies that compared the risk of death during TB treatment in patients with DM versus patients without DM, OR has a relatively stable value from 2008; however,

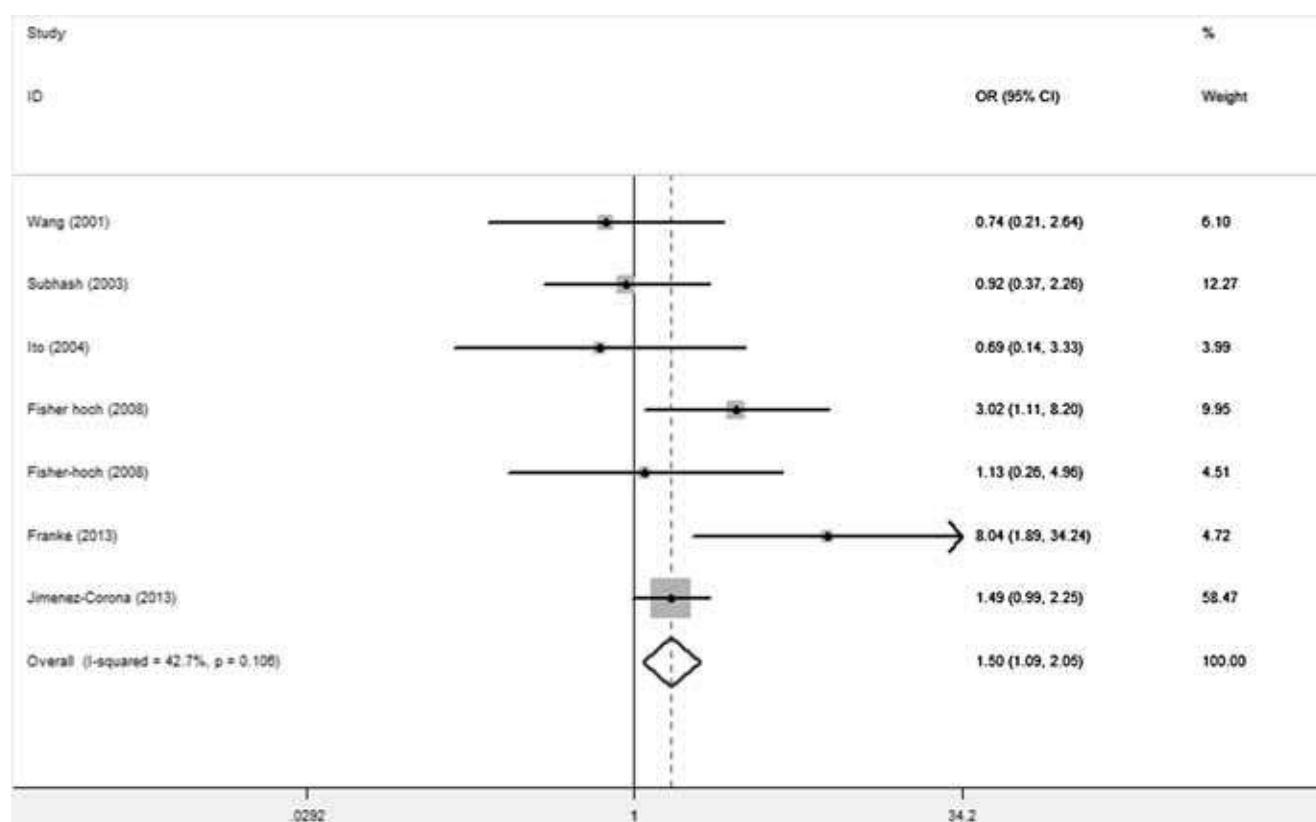
there is a growing trend in 2015. Begg's test suggested there existed publication bias ( $P = 0.001$ ) while Egger's test did not ( $P = 0.284$ ). The combined OR was not significantly altered by deleting each selected study sequentially, which indicated that our results were stable and robust.

As the growth of the year, the OR of this cumulative meta-analysis is more close to 1. There is no evidence for publication bias by Begg's test ( $P = 0.108$ ) or Egger's test ( $P = 0.097$ ). The study of Chiang [39] has a large sample size, so statistical heterogeneity is 63.8 % with this study and 54 % without it.

The 95 % confidence intervals of research before 2013 were all across 1; it means researchers find it meaningless.

**Fig. 18** OR and 95 % CI of drug-resistant recurrent disease on cumulative meta-analysis





**Fig. 19** OR and 95 % CI of drug-resistant recurrent disease

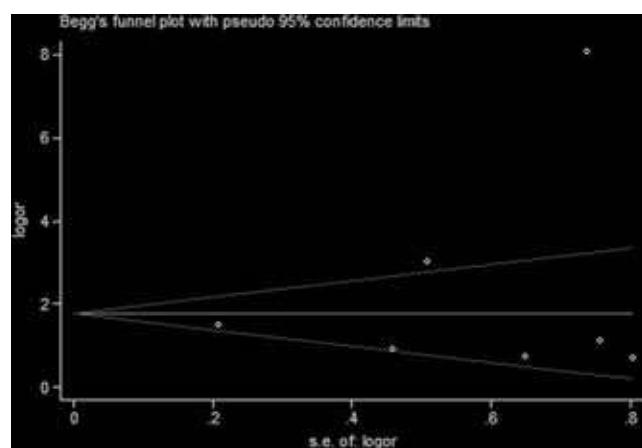
The effects pooled OR was 1.50 (95 % CI, 1.09 to 2.05), which determines that DM increases the risk. We did not find evidence for publication bias by Begg's test ( $P = 0.230$ ) or Egger's test ( $P = 0.504$ ). The statistical heterogeneity is 1 % without the study of Franke [25].

This cumulative meta-analysis highlights the need for large-scale prospective studies with appropriate study design, prospective diagnoses of diabetes, control for confounding factors, and clear TB outcomes to further clarify the strength of the associations. Today, about a third of

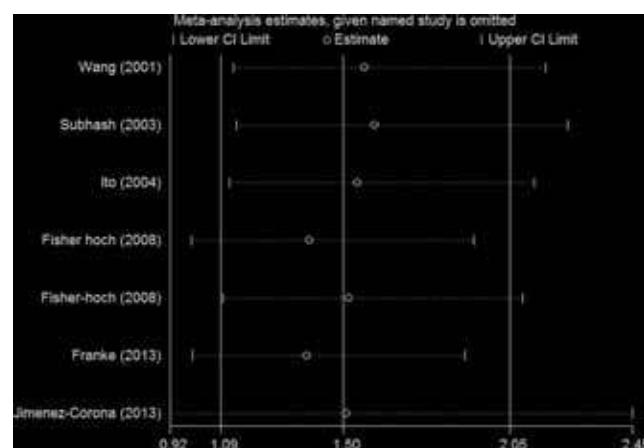
the world's population is latently infected with *M. tuberculosis*. And WHO has identified the progression of infection into rampant, contagious disease as a pressing challenge.

This is especially urgent in developing countries, where tuberculosis claims 2–3 million lives every year [66].

We also recommend further studies investigating how TB risk varies by type, duration, and severity of DM to have a more thorough understanding of the association that could be translated to a clear public health message.



**Fig. 20** Funnel plot for drug-resistant recurrent disease



**Fig. 21** Sensitivity analysis for drug-resistant recurrent disease

**Table 2** Characteristics of included studies for the association between DM and TB outcomes

Author	Type of study	Country	Type of TB	Total (n)	Population with DM (N)	Outcomes		DM definition		
						Sputum culture conversion 2–3 months	Failure and death	Death	Relapse	DR recurrence
Bashar [12]	Retrospective case-control study	USA	Undifferentiated TB	155	50		✓			Medical records
Centis [13]	Prospective cohort study	Italy	Undifferentiated TB	906	40	✓	✓			Medical records
Subhash [14]	Retrospective cohort study	India	Undifferentiated TB	361	72					$FBG > 140 \text{ mg/dL}$ , medical records and DM medication or diet
Singla [15]	Retrospective cohort study	Saudi Arabia	Pulmonary tuberculosis	692	187	✓	✓	✓	✓	Medical records
	Retrospective study	India	Pulmonary TB	190	92	✓				Medical records, FBG
Bana Rekha [16]	Retrospective analysis of 3 concurrent studies	Indonesia	tuberculosis (TB)	594	94	✓	✓			International criteria
Alisjahbana [17]	Prospective cohort study	Texas/Mexico	Undifferentiated TB	2878	688	✓				Self-report
Fisher-Hoch [18]	Retrospective cohort study	Maryland	Tuberculosis	197	42	✓				Medical records
Dooley [19]	Retrospective cohort study	Taiwan	Pulmonary tuberculosis	217	74	✓	✓	✓	✓	Medical records
Wang [20]	Retrospective cohort study	China	Pulmonary TB	2141	203					$FBG \geq 126 \text{ mg/dL}$
Zhang [21]	Retrospective cohort study	Turkey	Undifferentiated TB	156	78					Medical records
Tatar [22]	Retrospective cohort study	Taiwan	Pulmonary TB	1127	241					Medical records
Chiang [23]	Prospective cohort study	Taiwan	Pulmonary tuberculosis	438	129					Medical records
Chang [24]	Prospective cohort study	Peru	Multidrug-resistant (MDR)	671	369					Medical records
Franke [25]	Retrospective cohort study	Southern Mexico	<i>Mycobacterium tuberculosis</i>	1262	374	✓	✓	✓	✓	Glucose levels $\geq 126 \text{ mg/dL}$ in fasting samples or $\geq 200 \text{ mg/dL}$ for random samples were considered diagnostic
Jiménez-Corona [26]	Prospective cohort study	Tanzanian	<i>Mycobacterium tuberculosis</i>	1250	197	✓	✓	✓	✓	Medical records
Daniel [27]	Case-control study	China	<i>Mycobacterium tuberculosis</i>	1589	189	✓	✓	✓	✓	International Diabetes Federation 2011
Mi [28]	Retrospective cohort study	Thailand	<i>Mycobacterium tuberculosis</i>	227	37	✓	✓	✓	✓	(1) Fasting plasma glucose levels $\geq 126 \text{ mg/dL}$ after fasting for at least 8 h on two occasions and/or (2) random plasma glucose levels $\geq 200 \text{ mg/dL}$
Duangrithi [29]	Prospective cohort study									
Sulaiman [30]	Retrospective cohort study	Malaysia	<i>Mycobacterium tuberculosis</i>	1267	338					Medical records
Kang [31]	Cohort study	South Korea	Multidrug-resistant tuberculosis	1407	239	✓	✓	✓	✓	Medical records
Nandakumar [32]	Retrospective cohort study	India	<i>Mycobacterium tuberculosis</i>	3116	667	✓	✓	✓	✓	A blood glucose value of 0.126 mg for fasting blood

Table 2 (continued)

Author	Type of study	Country	Type of TB	Total (n)	Population with DM (N)	Outcomes		DM definition		
						Sputum culture conversion 2–3 months and death	Failure and death	Death	Relapse	DR recurrence
Reis-Santos [33]	A prospective cohort Study	Brazil	<i>Mycobacterium tuberculosis</i>	29,273	1797	✓				
	A retrospective cohort study	America	<i>Mycobacterium tuberculosis</i>	1428	147		✓			
Magee [34]	A cohort study	America	<i>Mycobacterium tuberculosis</i>	1852	86		✓			
Magee [35]	A prospective cohort study	India	<i>Mycobacterium tuberculosis</i>	245	96		✓			
Viswanathan [36]	A prospective cohort study	India	<i>Mycobacterium tuberculosis</i>	209	89		✓			
Surgeon [37]	A prospective cohort study	Kiribati	<i>Mycobacterium tuberculosis</i>	275	101		✓			
Cavanaugh [38]	Case-control study									
Chiang [39]	Cohort study	Taiwan	Pulmonary tuberculosis	1473	705		✓	✓		
Delgado-Sánchez [40]	Retrospective cohort study	Mexico	Tuberculosis	181,378	34,938	sugar (FBS) or 0.200 mg for random blood sugar (RBS) or for postprandial blood sugar (PPBS) during diabetic screening				
Behnaz [41]	Retrospective cohort study	Iran	Tuberculosis	211	20		✓			

Table 2 (continued)

Author	Type of study	Country	Type of TB	Total (n)	Population with DM (N)	Outcomes		DM definition
						Sputum culture conversion 2–3 months	Failure and death	
Restrepo [42]	A retrospective cohort study	Texas	<i>Mycobacterium tuberculosis</i>	1441	401	✓		Medical records
Restrepo [42]	A retrospective cohort study	Mexico	<i>Mycobacterium tuberculosis</i>	3441	607	✓		Medical records
Guang [43]	Prospective cohort study	China	Pulmonary tuberculosis	1128	182	✓		Medical records
Viswanathan [44]	Cohort study	India	Pulmonary tuberculosis	827	209		✓	Medical records
Ambrosetti [45]	Prospective cohort study	Italy	Undifferentiated TB	778	32			Medical records
Ambrosetti [46]	Prospective cohort study	Italy	Undifferentiated TB	838	50			Medical records
Ambrosetti [47]	Prospective cohort study	Italy	Undifferentiated TB	715	40			Medical records
Annamatsini [48]	Retrospective cohort study	Thailand	Pulmonary TB	226	117			Medical records
Blanco [49]	Retrospective cohort study	Canary islands, Spain	Pulmonary TB	98	14	✓		Medical records
Centis [50]	Prospective cohort study	Italy	Undifferentiated TB	1162	56			Medical records
Fielder [51]	Retrospective cohort study	USA	Pulmonary TB	174	22			Medical records
Guler [52]	Retrospective cohort study	Turkey	Pulmonary TB	306	44	✓		Medical records
Hara [53]	Retrospective cohort study	Japan	Pulmonary TB	624	112	✓		Medical records
Hasibi [54]	Retrospective cohort study	Iran	Disseminated TB	50	6			Medical records
Ito [55]	Retrospective cohort study	Japan	Undifferentiated TB	109	16			Medical records
Kitahara [56]	Retrospective cohort study	Japan	Pulmonary TB	520	71	✓		Medical records
Kourbatova [57]	Retrospective case-control study	Russia	Undifferentiated TB	460	20			Medical records
Maalej [58]	Retrospective case-control study	Tunisia	Pulmonary TB	142	60		✓	Medical records
Mboussa [59]	Retrospective cohort study	Republic of the Congo	Pulmonary TB	132	32	✓	✓	2 measurements of FBG $\geq 126$ mg/dL
Oursler [60]	Retrospective cohort study	USA	Pulmonary TB	139	18			Medical records
Pina [61]	Retrospective cohort study	Spain	Undifferentiated TB	1511	73			Medical records
Ponce-De-Leon [62]	Prospective cohort study	Mexico	Pulmonary TB	581	172	✓		Medical records ( $FBG \geq 126$ mg/dL, non-FBG $\geq 200$ mg/dL sensitivity analysis)
Vasankari [63]	Retrospective cohort study	Finland	Pulmonary TB	629	92			Treatment with DM medications
Wada [64]	Retrospective cohort study	Japan	Pulmonary TB	726	143	✓		Medical records
Wang [65]	Retrospective cohort study	Taiwan	Pulmonary TB	453	75		✓	Medical records

**Table 3** The heterogeneity of the included studies through sensitivity analysis

Author	Year	OR (95 % CI)	$I^2$ (%)	P value
Kithara	1994	1.62 (1.46, 1.81)	67	<0.00001
Hara	1996	1.59 (1.43, 1.78)	63	0.0005
Wada	2000	1.61 (1.44, 1.79)	64	0.0003
Alisjahbana	2007	1.65 (1.48, 1.84)	67	0.0001
Banu Rekha	2007	1.65 (1.48, 1.84)	66	0.0001
Blanco	2007	1.63 (1.46, 1.82)	68	<0.00001
Guler	2007	1.59 (1.43, 1.77)	58	0.003
Dooley	2009	1.65 (1.48, 1.84)	67	0.0001
Tatar	2009	1.64 (1.47, 1.83)	68	<0.00001
Jimenez-Corona	2013	1.67 (1.49, 1.88)	67	<0.00001
Daniel	2013	1.65 (1.48, 1.84)	66	0.0002
Mi	2013	1.59 (1.42, 1.77)	58	0.003
Duangrithi	2013	1.64 (1.47, 1.83)	68	<0.00001
Reis-Santos	2013	1.69 (1.47, 1.93)	68	<0.00001
Restrepo	2006	1.63 (1.46, 1.83)	68	<0.00001
Restrepo	2006	1.70 (1.51, 1.92)	66	0.0001
Ambrosetti	1995	2.1 (1.74, 2.54)	47	0.007
Ambrosetti	1996	2.06 (1.70, 2.49)	45	0.01
Ambrosetti	1997	2.14 (1.78, 2.59)	44	0.01
Gentis	1998	2.10 (1.74, 2.54)	47	0.007
Gentis	1999	2.12 (1.76, 2.55)	47	0.008
Mboussa	2003	2.04 (1.69, 2.46)	43	0.02
Ponce-De-Leon	2004	2.10 (1.73, 2.55)	47	0.007
Anunnatsiri	2005	2.11 (1.75, 2.55)	47	0.007
Chiang	2009	2.44 (1.96, 3.05)	38	0.03
Wang	2009	2.09 (1.73, 2.52)	47	0.008
Jimenez-Corona	2013	2.08 (1.72, 2.51)	46	0.008
Mi	2013	2.03 (1.68, 2.45)	42	0.02
Kang	2013	2.19 (1.80, 2.66)	45	0.01
Nandakumar	2013	2.08 (1.72, 2.51)	46	0.009
Magee	2014	2.15 (1.78, 2.60)	44	0.01
Viswanathan	2014	2.09 (1.74, 2.52)	46	0.010
Surgeon	2014	2.09 (1.73, 2.51)	44	0.01
Cavanaugh	2015	2.12 (1.76, 2.55)	46	0.008
Chiang	2015	2.13 (1.77, 2.58)	46	0.009
Behnaz	2015	2.04 (1.69, 2.46)	37	0.04
Guang	2015	2.05 (1.70, 2.47)	42	0.02
Chang	2011	2.05 (1.70, 2.47)	39	0.03
Kithara	1994	1.83 (1.62, 2.07)	71	<0.00001
Ambrosetti	1995	1.82 (1.61, 2.06)	71	<0.00001
Ambrosetti	1996	1.81 (1.60, 2.05)	71	<0.00001
Ambrosetti	1997	1.84 (1.62, 2.08)	71	<0.00001
Centis	1998	1.81 (1.60, 2.05)	71	<0.00001
Bashar	2001	1.81 (1.60, 2.05)	70	<0.00001
Centis	1999	1.83 (1.61, 2.07)	71	<0.00001
Fielder	2002	1.79 (1.58, 2.03)	69	<0.00001
Oursler	2002	1.80 (1.59, 2.04)	70	<0.00001
Mboussa	2003	1.81 (1.60, 2.05)	71	<0.00001

**Table 3** (continued)

Author	Year	OR (95 % CI)	$I^2$ (%)	P value
Ponce-de-leon	2004	1.86 (1.64, 2.12)	71	<0.00001
Kourbatova	2006	1.83 (1.62, 2.08)	71	<0.00001
Pina	2006	1.83 (1.61, 2.07)	71	<0.00001
Singla	2006	1.83 (1.62, 2.07)	71	<0.00001
Alisjahbana	2007	1.82 (1.61, 2.06)	70	<0.00001
Vasankari	2007	1.84 (1.62, 2.09)	71	<0.00001
Fisher-Hoch	2008	1.95 (1.71, 2.23)	69	<0.00001
Hasibi	2008	1.82 (1.60, 2.06)	71	<0.00001
Chiang	2009	1.88 (1.64, 2.14)	71	<0.00001
Dooley	2009	1.82 (1.61, 2.07)	71	<0.00001
Maalej	2009	1.82 (1.61, 2.06)	71	<0.00001
Tatar	2009	1.82 (1.61, 2.06)	71	<0.00001
Wang	2009	1.81 (1.60, 2.05)	71	<0.00001
Mi	2013	1.82 (1.61, 2.06)	71	<0.00001
Duangrithi	2013	1.82 (1.61, 2.06)	71	<0.00001
Sulaiman	2013	1.85 (1.63, 2.10)	71	<0.00001
Kang	2013	1.87 (1.65, 2.13)	71	<0.00001
Nandakumar	2013	1.81 (1.60, 2.05)	71	<0.00001
Reis-Santos	2013	1.95 (1.71, 2.23)	70	<0.00001
Magee	2014	1.86 (1.64, 2.1)	69	<0.00001
Magee	2014	1.82 (1.60, 2.06)	71	<0.00001
Surgeon	2014	1.83 (1.62, 2.07)	71	<0.00001
Cavanaugh	2015	1.82 (1.61, 2.06)	71	<0.00001
Chiang	2015	1.61 (1.41, 1.83)	38	0.02
Wada	2000	1.71 (1.27, 2.31)	54	0.04
Mboussa	2003	1.78 (1.32, 2.41)	66	0.007
Singla	2006	1.87 (1.39, 2.51)	69	0.004
Maalej	2009	1.83 (1.37, 2.46)	67	0.006
Zhang	2009	1.62 (1.18, 2.22)	56	0.03
Jimenez-Corona	2013	2.29 (1.60, 3.27)	62	0.01
Kang	2013	2.11 (1.51, 2.89)	62	0.01
Viswanathan	2012	1.97 (1.42, 2.74)	68	0.004
Wang	2001	1.57 (1.13, 2.17)	46	0.1
Subhash	2003	1.60 (1.14, 2.24)	46	0.1
Ito	2004	1.54 (1.12, 2.13)	47	0.09
Fisher-Hoch	2008	1.38 (0.99, 1.93)	40	0.14
Fisher-Hoch	2008	1.52 (1.10, 2.09)	52	0.07
Franke	2013	1.38 (1.00, 1.90)	1	0.41
Jimenez-Corona	2013	1.51 (0.92, 2.45)	52	0.06

## Conclusions

This study reports that diabetes is associated with an increased risk of the combined endpoint of failure and death, death during TB treatment, and relapse. It is the first study to our knowledge that quantifies the associations based on a systematic review of the literatures. The implications of the negative impact of DM on TB outcomes include poor individual

outcomes, increased risk of secondary transmission, and increased incidence of TB disease. Considering the increasing burden of DM, particularly in areas with highly prevalent TB, TB control programs will need to expand efforts to focus on treatment and monitoring of patients with DM and TB disease.

#### Compliance with ethical standards

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REVIEW ARTICLE

# Pharmacogenetics and personalized treatment of type 2 diabetes mellitus

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**Abstract** Type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases in the world. An important difference in effectiveness and toxicity of hypoglycemic agents has been associated with the presence of genetic variants in people with T2DM. We conducted a literature review up November 2015 by combining keywords type 2 diabetes mellitus, hypoglycemic agents and pharmacogenetics (PKG). Metformin, sulfonylureas, and meglitinide drugs are widely used for the T2DM treatment, although new drugs in combination with metformin are administered. Genetic variants in proteins that function as carriers, channels, or metabolizing enzymes affect both the pharmacokinetics and pharmacodynamics of these agents. Significant progress in T2DM's pharmacogenetics has been made; however, more studies involving a larger number of patients from different ethnic groups must be done. Furthermore, patients with T2DM generally are complex patients receiving hypolipidemic and hypotensive medications. Drug-drug interaction studies between these drugs must be done to really know the contribution of each polymorphism in drug effectiveness and/or toxicity.

**Keywords** Hypoglycemic agents · Polymorphisms · Effectiveness · Toxicity

## Introduction

Currently, there are eight categories of hypoglycemic agents for T2DM treatment: (1) sulfonylureas (SU), (2) meglitinides, (3) biguanides, (4) thiazolidinediones (TZDs), (5) alpha-glucosidase (AG) inhibitors, (6) dipeptidyl peptidase-4 (DPP4) inhibitors, (7) glucagon-like peptide-1 (GLP1) analogs, and (8) sodium-glucose cotransporter-2 (SGLT2) inhibitors.

Despite the variety of drugs available for the T2DM treatment, many patients do not achieve clinical goals or manifest adverse effects due to the presence of another illness, lack of treatment adherence, drug-drug interaction, or by the presence of genetic variants. Genetic polymorphisms of metabolizing enzymes, transporters, receptors, and other therapeutic targets can affect the absorption, distribution, metabolism, and elimination of drugs that can lead to interindividual differences in drug efficacy and/or toxicity.

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## Methods

A PubMed systematic search of items that involved the words “polymorphism,” “type 2 diabetes mellitus,” “effectiveness,” “toxicity,” “pharmacogenetics,” and “pharmacogenomics” until November 2015 was done. We select those polymorphisms that were associated with toxicity or decreased response to hypoglycemic agents in patients with T2DM. In addition, the polymorphism selected should contribute to one of these two features, and the observed effect should be repeated by other studies (except for those drugs which only

one polymorphism has been reported to cause toxicity or ineffectiveness).

## Overview of hypoglycemic drugs

### Sulfonylureas (SU)

Between the secretagogues, the SUs stimulate insulin secretion by pancreatic beta cells.

SUs bind to their receptors, which leads to cell depolarization and insulin release. The main mechanism of action is closure ATP-sensitive K channels present in beta cells, and therefore, insulin release [1]. ATP-sensitive K channels from beta cell form a complex of two proteins: one pore-forming subunit (Kir6.2) encoded by KCNJ11 gene and a drug binding subunit (SUR1) encoded by ABCC8 gene. Therefore, investigation of variants in ABCC8 and KCNJ11 genes that affect drug response should be studied.

The E23K polymorphism (rs5219) in KCNJ11 gene is associated with T2DM development, as well as an increased risk of SU therapeutic failure [2]. A study has found that carriers of K variant have a better therapeutic response to gliclazide [3]. Holstein et al. suggest that patients with T2DM carrying the K variant have reduced SU's therapy response [4].

Another variant, S1369A, produced by a single nucleotide polymorphism (SNP) in ABCC8 gene (rs757110) influenced the hypoglycemic efficacy of SU in Chinese patients, where A carriers were more sensitive to gliclazide [5].

Genetic variants involved in the SU's pharmacodynamics are important in the effectiveness, mainly the gene transcription factor 7-like 2 (TCF7L2) encoding a transcription factor (TCF-4), is involved in the cellular differentiation and

proliferation [6]. The TCF7L2 gene is related to the risk of developing T2DM [7]. Interestingly, Javorski et al. analyzed patients with the rs7903146 polymorphism, which showed a greater gliclazide effect in those carriers with the CC genotype compared with those belonging to the group of CT + TT genotypes [8].

One study suggested that the magnitude of the fasting plasma glucose (FPG), decreased after 6 months of SU's plus metformin treatment in patients with T2DM, was associated with the rs163184 variant (T/G) presence in the potassium channel voltage-gated KQT-like subfamily Q member 1 (KCNQ1) gene [9]. The SU's FPG response was significantly lower in carriers of the risk GG genotype.

Furthermore, the R variant for rs1801278 in the IRS-1 gene encoding the insulin receptor substrate-1 (a component of the insulin signaling cascade) was associated with an increased risk of SU's failure due to uncontrolled hyperglycemia despite combined sulfonylurea and metformin treatment [10].

Furthermore, the SUs are metabolized primarily by cytochrome P450 enzyme complex (CYP450), the CYP2C9 isoform. There are two main allelic variants, CYP2C9\*2 (R144C) (rs1799853) and CYP2C9\*3 (I359L) (rs105791), which are associated with elevated SUs serum levels. Lower dose of tolbutamide was necessary to regulate the levels of serum glucose for those carriers with the CYP2C9\*3 variant compared to patients with wild CYP2C9 genotype (CYP2C9\*1). Moreover, a study by Kirchheimer et al. showed that the CYP2C9\*3 variant was associated with a decreased glyburide clearance [11]. Different studies in several populations (Korean, Finnish, American, Japanese, and Chinese) demonstrated that patients with the CYP2C9\*3 allele had higher SU's area under the curve (AUC) than those with CYP2C9\*1 allele [12–17]. Polymorphisms associated with SU response are summarized in Table 1.

**Table 1** Pharmacogenetic of SU drugs

Reference	Gene	rs number	Drug	Effect
Sesti [2]	KCNJ11	5219	Glibenclamide	K allele is associated to secondary SU failure.
Javorsky [3]			Gliclazide	K allele showed better therapeutic response.
Holstein [4]			Glimepiride or glibenclamide	K allele showed worse therapeutic response.
Zhang [5]	ABCC8	757110	Gliclazide	A carriers were more sensitive to Gliclazide, had more diminution of HbA1c levels.
Javorsky [8]	TCF7L2	7903146	Gliclazide, glimepiride, glibenclamide, glipizide	C allele showed better Gliclazide therapeutic effect
Scroner [9]	KCNQ1	163184	Gliclazide, glimepiride, glibenclamide, glipizide	GG genotype carriers showed significantly lower SU FPG response.
Sesti [10]	IRS-1	1801278	Glibenclamide	R allele is associated with increased risk of secondary failure.
Kirchheimer [11]	CYP2C9	1057910	Tolbutamide	*3 is associated with reduced Glyburide clearance.
Becker [12]			Glyburide	*3 carriers needed a lower dose of Tolbutamide in order to regulate glucose levels.
Shon [13]			Chlorpropamide	*3 allele showed higher SU AUC than *1 allele.
Niemi [14]			Glyburide, Glimepiride	*3 allele showed higher SU AUC than *1 allele.
Suzuki [15]			Glimepiride	*3 allele showed higher SU AUC than *1 allele.
Tan [16]			Glypizide	*3 allele showed higher SU AUC than *1 allele.
Lee [17]			Tolbutamide	*3 allele showed higher SU AUC than *1 allele.

## Meglitinides

The mechanism of action of meglitinides is by stimulating the pancreas and subsequent insulin release. Blockage of potassium channels leads to beta cell depolarization and this results in insulin release [18]. Repaglinide and nateglinide are fast prandial insulin releasers [19]. Lower risk of hypoglycemia makes these agents an attractive option for some elderly patients, in particular when other agents may be contraindicated [20].

Nateglinide is eliminated via hepatic transformation and subsequent renal tubular secretion. According to in vitro studies, approximately 70 % of the intrinsic nateglinide's clearance may be mediated by CYP2C9. Kirchheimer et al. showed that carriers of the CYP2C9\*3 variant significantly reduced oral nateglinide clearance.

Studies in a Chinese population with healthy individuals showed that the CYP2C9\*3 and the rs4149056 (T/C) polymorphisms in solute carrier organic anion transporter family member 1B1 (SLCO1B1) may affect the nateglinide pharmacokinetics, with higher AUC in subjects with these variants [21]. Other studies showed that the C allele carriers had increased AUC compared to T allele carriers when patients were treated with repaglinide [22, 23].

For its part, the repaglinide is metabolized by CYP2C8, and according to clinical studies, the carriers of CYP2C8\*3 (rs11572080 + rs10509681) have higher clearance than those with wild genotype (CYP2C8\*1) [22].

The presence of the variant \*18 (rs28371759) in the CYP3A4 influences some repaglinide pharmacokinetic parameters such as lower mean elimination rate constant and higher half-life than those with normal CYP3A4\*1 [24].

Another transporter involved in the therapeutic efficacy of repaglinide is the solute carrier family 30 (zinc transporter) member 8 (SLC30A8). The T allele of rs13266634 and the A allele of rs16889462 for these gene variants are associated with better repaglinide's response [25].

Repaglinide levels may be influenced by the presence of gene variants in the multidrug resistance (MDR1). Three SNPs were evaluated in a Chinese population, and a unique variant, rs2032582 (G/T/A), was associated with variations in the pharmacokinetics of repaglinide, suggesting that the GT and TT carriers could be exposed to high repaglinide levels [26].

In relation to KCNJ11 gene, the E23K mutation is associated with a greater decrease of HbA1c levels in K carriers [27].

Also, Yu et al. [28] demonstrated that variations in the KCNQ1 gene in Chinese patients with DM2, and homozygous TT for the rs2237892 polymorphism, had lower glucose levels after repaglinide treatment, while those with the risk C allele for the variant rs2237895 was associated with a greater increase in both fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) levels. In turn, patients

with T2DM and the TT genotype for the rs290487 (in the gene TCF7L2), the treatment was more effective with respect to fasting insulin, triglycerides, and low-density lipoprotein cholesterol levels compared with those with CC or CT genotypes [27].

Finally, polymorphisms in insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) gene, such as rs1470579 and rs4402960 were associated with the development of T2DM with repaglinide's therapeutic efficacy on T2DM [29]. The effects of repaglinide's treatment at the FPG and PPG levels were reduced in patients with DC + AC genotypes compared with AA carriers for the rs1470579. However, patients with GT + TT genotypes showed a greater effect of repaglinide treatment in postprandial serum insulin levels (PINS) compared to subjects with the GG genotype for the rs4402960. Polymorphisms associated with Meglitinides response are summarized in Table 2.

## Biguanides

Metformin reduces hyperglycemia mainly by suppressing the production of glucose by hepatic gluconeogenesis [30]. The exact mechanism of action of metformin is not known.

This drug causes few side effects when prescribed correctly and has been associated with lower risk of hypoglycemia [31]. Lactic acidosis can be a serious problem in the case of overdose and in persons with contraindications, but usually, there are no significant risks [32].

Unlike most drugs, metformin does not undergo hepatic transformation and is not metabolized [33]. However, it is transported to and from the cells and organs [34].

Several pharmacogenetic studies have reported the importance of gene coding for transporters such as organic cation transporter 1(OCT1) (SLC22A1 gene), organic cation transporter 2 (OCT2) (SLC22A2 gene), organic cation transporter 3 (OCT3) (SLC22A3 gene), multidrug and toxin extrusion 1 (MATE1) protein (SLC47A1 gene), and multidrug and toxin extrusion2 (MATE2) protein (SLC47A2 gene). Some polymorphisms in the SLC22A1 gene were associated with metformin's side effects (rs628031, rs36056065) [35], with variations in pharmacokinetic parameters of metformin (greater AUC and higher maximum plasma concentration ( $C_{max}$ )) (rs12208357, rs34130495, rs72552763, rs34059508) [36] and clinical efficacy of metformin (rs628031, rs12208357, rs2282143, rs35191146, rs34130495, rs34059508, rs461473) [37, 38].

One of metformin transporters, the OCT2 protein is expressed specifically in the kidney and plays a critical role in renal secretion. The rs201919874 (C/T), rs145450955 (C/T), and rs316019 (G/T) variations exhibited decreased biological function leading to increased plasma concentrations of metformin [39].

The protein encoded by the SLC47A1 gene is responsible for metformin excretion in bile and urine, respectively. The

**Table 2** Pharmacogenetic of meglitinides drugs

Reference	Gene	rs number	Drug	Effect
Kirchheimer [20] Cheng [21]	CYP2C9	1057910	Nateglinide	*3 variant showed significantly reduced oral nateglinide clearance. *3 allele showed higher nateglinide AUC than *1 allele.
Cheng [21] Niemi [22] Kallikoski [23]	SLCO1B1	4149056	Nateglinide Repaglinide Repaglinide	C allele showed higher nateglinide AUC than T allele. C allele exhibited higher repaglinide AUC than T allele. C allele exhibited higher repaglinide AUC than T allele.
Niemi [22]	CYP2C8	11572080+10509681	Repaglinide	*3 allele showed lower repaglinide AUC than *1 allele.
Ruzilawati [24]	CYP3A4	28371759	Repaglinide	*18 displayed higher repaglinide half-life than those with *1 allele.
Huang [25]	SLC30A8	13266634  16889462	Repaglinide	T allele showed better repaglinide response on FINS and PINS than C allele. A allele showed enhanced repaglinide efficacy in FPG, PPG, and HbA1c than G allele.
Xiang [26]	ABCB1	2032582	Repaglinide	GT and TT genotypes had higher repaglinide AUC than GG and GA genotypes.
Yu [27]	KCNJ11	5219	Repaglinide	Patients with GA or AA genotype showed higher levels of FPG, PPG, and HbA1c compared with patients with GG genotype.
Yu [27]	TCF7L2	290487	Repaglinide	Patients with TT genotype showed enhanced repaglinide efficacy in FINS than CC or CT genotypes.
Yu [28]	KCNQ1	2237892	Repaglinide	TT homozygotes showed lower glucose levels following repaglinide treatment.
Huang [29]	IGF2BP2	1470579  4402960	Repaglinide	Patients with AC+CC genotypes showed reduced repaglinide efficacy in FPG and PPG compared with AA genotype. Patients with GT+TT genotypes exhibited enhanced repaglinide effect on PINS compared with GG genotype.

rs2289669 (G/A) was significantly associated with metformin response. For each minor A allele of rs2289669, HbA1c reduction was 0.30 % higher than in those with the G allele [40]. The variant rs2252281 (T/C) in the promoter of SLC47A1 gene is associated with a greater response to metformin in patients with T2DM measured as a relative change in HbA1c mean levels [41]. However, the rs12943590 (G/A) polymorphism in the SLC47A2 gene is associated with reduced metformin [41]. In another study, patients with diabetes who were AA homozygous showed a poorer response to metformin measured as a relative change in HbA1c compared with those with the G reference allele [42].

The ataxia telangiectasia mutated (ATM) locus includes chromosome 11 open reading frame 65 (C11orf65) gene associated with glycemic response to metformin, particularly the C allele for the rs11212617. This variant showed a greater decrease in HbA1c levels than those with the A allele [43]. Polymorphisms associated with Biguanides response are summarized in Table 3.

### Thiazolidinediones (TZD)

TZDs are a class of oral hypoglycemic drugs that improve glycemic control in patients with T2DM by improving insulin sensitivity. TZDs exert their hypoglycemic effects through a mechanism that involves activation of gamma isoform of the peroxisome proliferator-activated receptor (PPAR $\gamma$ ), a nuclear receptor. Activation by TZDs-induced PPAR $\gamma$  alters the

transcription of several genes involved in energy balance, metabolism of glucose and lipids, including those encoding lipoprotein lipase, a fatty acid transport protein, binding protein adipocyte fatty acids, fatty acyl-CoA synthase, malate dehydrogenase, glucokinase, and glucose transporter GLUT4 [44].

The TZDs safety (pioglitazone and rosiglitazone) are related to liver and cardiovascular problems, fluid retention, weight gain, and bone fractures. However, pioglitazone tends to be cardioprotective while rosiglitazone is cardiotoxic [45].

The PPAR $\gamma$ 2 has one common, not synonymous polymorphism, that is often found in the population, the P12A (rs1801282) [46]. Functional studies showed that the A allele has reduced DNA binding PPAR $\gamma$  response elements and reduced transcriptional activation in the presence of TZDs [47]. A study with obese postmenopausal women showed a greater reduction in glucose levels in subjects with the PA genotype than in those with the PP genotype [48]. Kang et al. found that patients with the PA genotype showed a better therapeutic response to rosiglitazone than those with genotype PP measured as decreased fasting plasma glucose and HbA1c levels [49].

Moreover, Hsieh et al. demonstrated that the decrease in FPG and HbA1c levels after pioglitazone treatment was significantly higher in subjects with the A allele (AP and AA) than those without the A allele (PP) [50]. According to this study, patients with the PA genotype had significantly higher serum triglyceride levels and differential PPG values compared to those with the PP genotype [51]. They also showed

**Table 3** Pharmacogenetic of metformin

Reference	Gene	rs number	Drug	Effect
Tarasova [35]	SLC22A1	628031	Metformin	A allele is associated to gastrointestinal side effects.
Tarasova [35]		36056065		8-bp insertion allele is associated to gastrointestinal side effects.
Shu [36]		12208357		C carriers showed higher metformin AUC than R carriers.
Yang [38]				C carriers showed higher HbA1c after metformin treatment than R carriers.
Shu [36]		34130495		S carriers showed higher metformin AUC than G carriers.
Yang [38]				S carriers showed higher HbA1c after metformin treatment than G carriers.
Shu [36]		72552763		Deletion carriers showed higher metformin AUC than wt carriers.
Yang [38]				Deletion carriers showed higher HbA1c after metformin treatment than M carriers.
Shu [36]		34059508		R carriers showed higher metformin AUC than G carriers.
Yang [38]				R carriers showed higher HbA1c after metformin treatment than G carriers.
Song [39]	SLC22A2	201919874	Metformin	CT carriers showed higher metformin AUC than CC carriers.
Song [39]		145450955		CT carriers showed higher metformin AUC than CC carriers.
Song [39]		316019		GT and TT carriers showed higher metformin AUC than GG carriers.
Becker [40]	SLC47A1	2289669	Metformin	A carriers showed more HbA1c reduction after metformin treatment than G carriers.
Stocker [41]		2252281		C carriers showed more HbA1c reduction after metformin treatment than T carriers.
Stocker [41]	SLC47A2	12943590	Metformin	GG and GA carriers showed higher HbA1c reduction after metformin treatment than AA carriers.
Choi [42]				GG carriers showed higher HbA1c reduction after metformin treatment than AA carriers.
Zhou [43]	C11orf65	11212617	Metformin	C carriers showed greater reduction of HbA1c than those with A allele carriers.

that the rs17584499 polymorphism (C/T) in the protein tyrosine phosphatase receptor type D (PTPRD) gene was associated with pioglitazone's therapeutic efficacy. Patients with CT and TT genotypes had significantly lower differential PPG values compared to those with the CC genotype.

A study by Makino et al. analyzed the polymorphism rs1862513 (C/G) in resistin gene in patients with T2DM and revealed that patients with the GG genotype but not with the CG correlated with a reduction in fasting plasma glucose and HOMA-IR compared to the CC genotype [52].

As mentioned above, polymorphisms in drug transporters have been studied and in this case, we want to show their relationship with the TZDs effectiveness. The R219K variant (rs2230806) in the ABCA1 gene was studied in patients with T2DM, and rosiglitazone's treatment response was evaluated after 48 weeks. Individuals homozygous RR showed a better response to treatment with rosiglitazone in terms of improved insulin sensitivity than the carriers of K minor allele [53].

Rosiglitazone and pioglitazone are metabolized in the liver by cytochromes, mainly by CYP2C8 and with lower CYP2C9 contribution [54]. Several polymorphisms not synonymous for CYP2C8 were observed, especially \*2 and \*3, both with decreased enzyme activity [54]. Some studies investigated the contribution of CYP2C8\*3 in patients treated with rosiglitazone. The mean AUC was lower in individuals homozygous CYP2C8\*3 compared to subjects who had the wild variant [55].

Some polymorphisms in the retinol binding protein 4 (RBP4) gene and the response to rosiglitazone treatment in T2DM Chinese patients was evaluated. Patients with the GG genotype for the rs3758539 (G/A) in RBP4, showed an enhanced efficacy of rosiglitazone expressed in FPG and FINS

as compared to the GA or AA genotypes. Yet, another polymorphism in the same gene, the rs10882283 (T/G), showed that in patients with TG or GG genotypes had higher efficacy than those in the TT group measured as HbA1c levels [56].

Kang et al. studied the effects of rosiglitazone on plasma glucose and adiponectin levels relative to common polymorphisms in the adiponectin gene (ACDC). There was a lower reduction in the level of fasting plasma glucose and HbA1c in GG carriers compared with other genotypes for the rs2241766 variant. With respect to the rs1501299 variant, it had less reduction in fasting plasma glucose level for the GG genotype than for other genotypes. A haplotype analysis showed reductions in HbA1c levels, and FPG were lower for the homozygous GG haplotype than the other haplotypes [57]. Polymorphisms associated with TZD response are summarized in Table 4.

#### **α-glucosidase (AG) inhibitors**

α-glucosidase inhibitors, including acarbose, are competitive inhibitors of membrane-bound intestinal α-glucosidases that hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine and thereby delay postprandial glucose absorption [58].

PPAR alpha (PPAR $\alpha$ ), a transcription factor of the nuclear receptor superfamily regulates fatty acid oxidation. Andrlionyte et al. showed a study where in the placebo group, the G allele of rs1800206 (C/G) increased the risk of diabetes and was associated with elevated levels of plasma glucose and insulin. However, in the acarbose group, subjects carrying the minor G allele for rs4253776 SNP and CC genotype for rs4253778, both in the of PPAR $\alpha$  gene, had an

**Table 4** Pharmacogenetic of thiazolidinedione drugs

Reference	Gene	rs number	Drug	Effect
Kang [49]	PPAR $\gamma$	1801282	Rosiglitazone	Patients with PA genotype showed better therapeutic response to rosiglitazone than patients with PP genotype.
Hsieh [50]			Pioglitazone	Patients with A allele showed better therapeutic response to pioglitazone than patients with P allele.
Pei [51]			Pioglitazone	Patients with A allele showed higher FPG and TG differential values after pioglitazone treatment than patients with P allele.
Pei [51]	PTPRD	17584499	Pioglitazone	Patients with CT+TT genotypes showed lower differential value of PPG compared to those with CC genotype.
Makino [52]	RETN	1862513	Pioglitazone	HbA1c reduction was correlated with GG genotype.
Wang [53]	ABCA1	2230806	Rosiglitazone	RR homozygotes showed a better response to rosiglitazone treatment than minor K allele carriers.
Stage [55]	CYP2C8	11572080+10509681	Rosiglitazone	*3 carriers showed lower rosiglitazone AUC than *1 carriers.
Zhou [56]	RBP4	3758539	Rosiglitazone	Patients with GG genotype showed better rosiglitazone efficacy in FPG and FINS compared with that in GA+AA genotype.
		10882283		Patients with TG+GG genotype showed better rosiglitazone efficacy in HbA1c level compared with that in TT genotype.
Kang [57]	ADIPOQ	2241766	Rosiglitazone	GG carriers showed a smaller reduction in FPG and HbA1c than carriers with other genotypes
		1501299		GG genotype showed lower reduction in FPG than in other genotypes

increased risk of diabetes [59]. In another study, subjects with PP genotype for rs1801282 in PPAR $\gamma$ 2 gene and S allele for rs8192678 in PPAR- $\gamma$  coactivator 1 alpha (PGC-1 $\alpha$ ) gene were associated with the conversion from impaired glucose tolerance to T2DM in the STOP-NIDDM trial [60].

#### Dipeptidyl peptidase-4 (DPP4) inhibitors

The DPP-4 or gliptins are a class of oral hypoglycemic agents blocking the DPP4 enzyme.

While glucagon raises blood glucose levels, DPP4 inhibitors reduce glucagon and glycemia levels. The DPP4-inhibitor action is increasing the incretins levels ("GLP-1" and gastric inhibitory polypeptide "GIP") [61], which inhibit the release of glucagon, which in turn increases insulin secretion, also decreasing gastric emptying and glycemia levels.

Adverse effects, including nasopharyngitis, headache, nausea, heart failure, hypersensitivity, and skin reactions have been observed in clinical studies [62].

Zimdahl et al. showed an association between the rs7903146 polymorphism in the TCF7L2 gene and the degree of response after linagliptin treatment. Patients with the CC genotype showed a greater reduction in HbA1c than those with the TT genotype [63].

The non-synonymous polymorphism rs147614497 (R623Q) is present in the DPP4 gene. In vitro studies showed that the R623Q led to decrease vildagliptin hydrolysis [64].

Other variants could be studied such as those in the CYP3A4/A5 genes because saxagliptin is metabolized largely by both cytochromes [62].

#### Glucagon-like peptide-1 (GLP1) analogs

GLP-1 is a natural peptide hormone, secreted from the intestine after a meal. GLP-1 stimulates insulin release (the incretin effect), suppresses glucagon release (which reduces hepatic gluconeogenesis), slows gastric emptying, and promotes satiety. The rs367543060 (T149M) in the GLP1 receptor (GLP-1R) has contributed to altering the response of GLP-1 in vitro [65].

Natural GLP1 has a short half-life, of only a few minutes, as a result of degradation by endopeptidases such as DPP4. GLP1 analogs, also known as incretin mimetic (exenatide and liraglutide), are a modified GLP1 and are resistant to degradation by DPP4 [66]. GLP1 analogs are given in combination with other treatments in the management of T2DM and is applied once daily (liraglutide) or twice daily (exenatide) by subcutaneous injection.

One of its biggest advantages over older insulin secretagogues, like SUs or meglitinides, the risk of causing hypoglycemia is much lower.

To date, only one study has shown an association between the presence of an SNP in the cannabinoid 1 receptor (CNR1) gene and liraglutide effectiveness. Those subjects without the A allele of the rs1049353 (G/A) showed improved cholesterol levels after weight loss in Spanish T2DM patients [67].

#### SGLT2 inhibitors

Inhibitors of glucose co-transporter sodium 2 (SGLT2) is a new drug class indicated only for the treatment of T2DM.

SGLT2 is a human protein that facilitates the reabsorption of glucose at the kidney. SGLT2 inhibitors block the reabsorption of glucose at the kidney, increase the excretion of glucose, and improve glycemic control and weight loss [68].

The advantages of this class of agents include modest weight loss, lower risk of hypoglycemia, and decreased blood pressure. However, use of these drugs are limited due to the high frequency of genital fungal infections, and less common side effects are hypotension, dizziness, and worsening renal function [69].

Over 50 different mutations in the SLC5A2 gene encoding SGLT2 protein were found, and these mutations could lead to renal glycosuria [70]. Enigk et al. suggest the realization of pharmacogenomic studies to determine if the efficacy of treatment with SGLT2 inhibitors could be affected by the presence of polymorphisms in the SLC5A2 gene [71]. For the moment, no studies have been published of patients with DM2 about genetic variants and response to SGLT2 inhibitors. Meanwhile, dapagliflozin, an SGLT2 inhibitor, is mainly metabolized through uridine diphosphate-glucuronosyltransferase 1-9 (UGT1A9) to give its major inactive metabolite, dapagliflozin 3-O-glucuronide; therefore, mutations in this gene could affect the efficacy of treatment [72]. Polymorphisms associated with α-glucosidase Inhibitors, Dipeptidyl peptidase-4 inhibitors, Glucagon-like peptide-1 analogues and SGLT2 inhibitors responses are summarized in Table 5.

## Discussion

In patients with DM2, fulfilling the objectives of glycemia and HbA1c requires striking a balance between age, comorbidities, and risk of hypoglycemia. The American Association of Clinical Endocrinologists suggests achieving values lower than 6.5 % of HbA1c for most patients, which significantly reduces the risk of developing nephropathy [73]. It has also been found that maintaining lower HbA1c levels reduces the risk of developing cardiovascular disease. According to this, glycemic control must be individualized. In newly diagnosed patients with type 2 diabetes and without cardiovascular disease, HbA1c levels between 6.0 and 6.5 % must be desirable, which ensures minimal risk of hypoglycemia or other adverse consequences. A broader range of HbA1c levels can be handled in elderly patients or those at risk of hypoglycemia. Tolerance may be higher (7–8 % HbA1c) for patients with a history of severe hypoglycemia, kidney disease, or macrovascular complications. Therefore, the choice of drug therapy should consider the therapeutic goal to be achieved, in addition to age and other factors such as risks of adverse effects of each treatment.

In the Glycemic Control Algorithm proposed by the American Association of Clinical Endocrinologists [73], a hierarchy of hypoglycemic agents is suggested. Following this algorithm, we propose the analysis of polymorphisms associated with lack of drug efficacy and/or toxicity of agents

**Table 5** Pharmacogenetic of α-glucosidase inhibitors, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 analogs, and SGLT2 inhibitors

Reference	Gene	rs number	Drug	Effect
<b>α-glucosidase inhibitors</b>				
Andrulionyte [59]	PPAR $\alpha$	4253776 4253778	Acarbose	G carriers showed an increased risk of diabetes. Patients with the CC genotype showed an increased risk of diabetes.
Andrulionyte [60]	PPAR $\gamma$	1801282	Acarbose	PP genotype for rs1801282 and S allele for rs8192678 were associated with the conversion from impaired glucose tolerance to T2DM.
Andrulionyte [60]	PPAR $\gamma$ C1A	8192678	Acarbose	PP genotype for rs1801282 and S allele for rs8192678 were associated with the conversion from impaired glucose tolerance to T2DM.
<b>Dipeptidyl peptidase-4 inhibitors</b>				
Filippatos [62]	CYP3A4	Undescribed yet	Saxagliptin	Unknown
Filippatos [62]	CYP3A5	Undescribed yet	Saxagliptin	Unknown
Asakura [64]	DPP4	147614497	Vildagliptin	R623Q mutation resulted in a decrease in vildagliptin-hydrolyzing activity.
Zimdahl [63]	TCF7L2	7903146	Linagliptin	Patients with TT genotype showed reduced HbA1c response compared with CC patients after linagliptin treatment.
<b>Glucagon-like peptide-1 analogs</b>				
De Luis [67]	CNR1	1049353	Liraglutide	A carriers showed an improvement in insulin resistance secondary to weight loss after liraglutide treatment.
<b>SGLT2 inhibitors</b>				
Enigk [71]	SLC5A2	9934336	None	G-allele was associated with increased 30-min plasma glucose, 120-min insulin concentrations and AUC120min (glucose) during oral glucose tolerance.
Kasichayanula [72]	UGT1A9	Someone	Dapagliflozin	Unknown

delivered to a patient; hence, when these variants are detected, we should, ideally, try to change to another drug for which the patient does not present the unfavorable variant. We claim now that it is not inconsistent with what has mentioned above; we believe that it is necessary to make many previous studies to determine, more precisely, which of the polymorphisms proposed today will have clinical use tomorrow. According to the American Association of Clinical Endocrinologists, the drug hierarchy is as follows: (1) metformin, (2) GLP1 agonists, (3) SGLT2 inhibitors, (4) DPP4 inhibitors, (5) TZD, (6) alpha-glucosidase inhibitors, and (7) SU/meglitinides. Therefore, genes and polymorphisms that could be studied are classified according to the previous hierarchy: (1) SLC22A1, SLC22A2, SLC47A1, SLC47A2, etc. (Table 3); (2) CNR1 (Table 5); (3) SLC5A2, UGT1A (Table 5); (4) CYP3A4, CYP3A5, DPP4, TCF7L2 (Table 5); (5) PPAR $\gamma$ , PTPRD, RETN, ABCA1, CYP2C8, etc. (Table 4); (6) PPAR $\alpha$ , PPAR $\gamma$ , PPAR $\gamma$ C1A (Table 5); and (7) KCNJ11, ABCC8, TCF7L2, KCNQ1, CYP2C9 for SU (Table 1) and CYP2C9, SLCO1B1, CYP2C8, CYP3A4, SLC30A8, etc. (Table 2).

This kind of suggestion would apply to monotherapy treatment (patients with <7.5 % HbA1c at baseline) and also to those requiring double or triple therapy (patients with >7.5 % HbA1c at baseline). One possible application described throughout this review involves the use of information gathered from genetic variants in the SLC22A1 gene, which codifies a carrier entering the blood circulating metformin to the hepatocyte. In our research, we studied a group of patients with type 2 diabetes who were receiving 2500 mg/day of metformin (maximum recommended dose) and analyzed them to detect the presence of five polymorphisms in the SLC22A1 gene (rs12208357, rs2282143, rs35191146, rs34130495, and rs34059508; all these variants lead to reduced transporter activity). After 3 months of metformin treatment with 2500 mg/day, most patients receiving this dose did not achieve the target HbA1c levels. When comparing this therapeutic failure with the variants in the SLC22A1 gene, we discovered that most of them had more than one polymorphism in the SLC22A1 gene, which could significantly affect the drug income into the hepatocyte, significantly decreasing its therapeutic action. For this group of patients, we proposed to use another hypoglycemic drug (SU, meglitinides, TZDs, AG inhibitors, DPP4 inhibitors, GLP-1 analogs, and SGLT2 inhibitors), seeking the most effective treatment, measured as HbA1c level. To attain this, there are two options, (A) polymorphism analysis, associated with the new drug (effectiveness/toxicity) or (B) directly test the new drug and wait 3 months and see whether HbA1c values have reached the therapeutic target. The use of the first or the second option will depend on several factors. Currently, there are no data from clinical studies in which a large number of patients from different ethnicities were analyzed to determine what

polymorphisms must be necessarily analyzed. This could be an expensive study, and today, we are not sure whether the analysis could be useful. This claim does not overlook the need to conduct this kind of studies. The second option seems to be more viable and less expensive, and it is what indeed happens. The analysis involves treatment with one hypoglycemic drug; yet, we believe it can be extrapolated when therapeutic goals have not been fulfilled and the recommending of the beginning of a combination therapy of two or three drugs.

Such recommendations are not new and have been proposed for other diseases and drug treatment associated with them. Today, FDA and EMEA show a list of drug/gene variants that can/should be analyzed prior to the use of a particular drug. Examples include (drug-polymorphism) abacavir-HLA-B\*5701, capecitabine-DPYD\*2A, irinotecan-UGT1A1\*28, cetuximab-KRAS exon 12 and 13, and clopidogrel-CYP2C19\*2 and \*3. Therefore, the analysis of polymorphisms in genes that may affect the effectiveness/toxicity of a given hypoglycemic drug could be useful in the pharmacological treatment of patients with DM2 while taking into account the importance of other non-genetic factors including treatment adherence, physical activity, and appropriate diet.

## Conclusion

As mentioned above, it is clear that there are numerous variations in patient's DNA that could contribute to variability in both drug's effectiveness and toxicity.

Different polymorphisms analyzed may affect the pharmacokinetics and pharmacodynamics of different hypoglycemic drugs; in fact, variants in genes encoding channels (KCNJ11, ABCC8, ABCB1, ABCA1, etc), transporters (SLCO1B1, SLC22A1, SLC22A2, SLC22A3, SLC5A2, etc), receptors (PPAR $\gamma$ , PPAR $\alpha$ , etc), and metabolizing enzymes (CYP2C9, CYP2C8, CYP3A5, CYP3A4, UGT1A9, etc) have been shown.

Then, it will be necessary to conduct studies with a larger number of patients and with different ethnic background to validate the results published. In fact, there are new hypoglycemic drug categories and new drugs, and there is currently no association with known polymorphisms to influence their effectiveness or toxicity, probably because no association studies were done.

Advances in pharmacogenetics of T2DM are significant although a clinical application of these depends primarily on the increase in the number of studies to validate the results obtained in different populations. Unfortunately, for many of the polymorphisms analyzed, only few studies have been published or were conducted with a small number of patients, making it difficult to extrapolate these results to a larger population or to be clinically used.

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#### Compliance with ethical standards

**Ethical responsibilities of authors** The manuscript has not been submitted to any other journals, has not been published previously, and has not been fabricated or manipulated.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Informed consent** The informed consent of each patient was collected in each of the cited articles.

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ORIGINAL ARTICLE

# Predictive risk modelling for early hospital readmission of patients with diabetes in India

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**Abstract** Hospital readmission is an important contributor to total medical expenditure and is an emerging indicator of quality of care. The goal of this study is to analyze key factors using machine learning methods and patients' medical records of a reputed Indian hospital which impact the all-purpose readmission of a patient with diabetes and compare different classification models that predict readmission and evaluate the best model. This study classified the patients into two different risk groups of readmission (Yes or No) within 30 days of discharge based on patients' characteristics using 2-year clinical and administrative data. It proposed an architecture of this prediction model and identified various risk factors using text mining techniques. Also, groups of consistently occurring factors that influence readmission rates were revealed by associative rule mining. It then evaluated the

classification accuracy using five different data mining classifiers and conducted cost analysis. Out of total 9381 records, 1211 (12.9 %) encounters were found as readmissions. This study found that risk factors like hospital department where readmission happens, history of recent prior hospitalization and length of stay are strong predictors of readmission. Random forest was found to be the optimal classifier for this task using the evaluation metric area under precision-recall curve (0.296). From the cost analysis, it is observed that a cost of INR 15.92 million can be saved for 9381 instances of diabetic patient encounters. This work, the first such study done from Indian Healthcare perspective, built a model to predict the risk of readmission within 30 days of discharge for diabetes. This study concludes that the model could be incorporated in healthcare institutions to witness its effectiveness. Cost analysis shows huge savings which is significant for any healthcare system especially in developing countries like India.

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**Keywords** Predicting hospital readmission rates · Medical data analysis · Machine learning · Association rule mining · Feature selection analysis · Cost-sensitive analysis

## Introduction

A readmission is the next subsequent admission of a patient as an acute admission within a defined period of time [1]. Much focus is placed on readmission within 30 days, often called early readmission, because it represents poor healthcare quality and potentially avoidable cost [2, 3]. Furthermore, the rate of readmission is chosen as one of the grounds for measuring the quality of health care in the National Quality Forum of 2008 in the USA. US Centers for Medicare and Medicaid have specified readmission as a reason for the excessive

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medical expenses [4]. Readmissions would consume resources for a given population in a time frame, increasing the cost for overall medical treatment. The US Medicare Payment Advisory Commission has stated that \$12 billion is spent on preventable readmissions annually, and a study estimated readmission cost of Medicare patients to be \$17.4 billion per annum [2]. Hence, reducing cost associated with readmission is very important to any nation especially developing ones.

The concept of 30-day unplanned readmission in India is in its very nascent stage and much of the data is currently evolving. Several predictive models have been developed for readmissions especially in the USA where hospitals are penalized for unplanned 30-day readmissions. However, not much work has been done in Indian hospitals to predict readmissions because the lack of digitized data serves as a barrier, and there is no incentive resulting from readmission-based payment penalties. But, the health insurance companies in India are looking at the readmission metrics pretty vigilantly and are expected to come with strong recommendations in the near future [5]. Predictive models based on a specific patient subpopulation are of more value than models based on a general cohort, because the specific patients' characteristics related to certain diseases may play important roles in model construction. Therefore, this work has been limited to readmission of patients with diabetes as diabetes is one of the most prevalent diseases and a major health hazard in developing countries like India.

The enormous growth of the digital health data (called Big Data) generates opportunities for greater patient insights that may help lower healthcare costs in India while providing better health care access [6, 7]. This proposed solution is scalable to any volume, variety, and velocity of data resulting in more efficient and faster results and insights. This research study aims to build a predictive model that can classify patients with diabetes who might get readmitted within 30 days by analyzing data pertaining to their medical history. The model also finds statistically significant patterns in the features of patients' medical records which can determine his early hospital readmission. This study proposes an architecture of early readmission risk prediction modelling process and explains its various steps. An extensive experimental study including cost analysis is conducted using the real-world dataset of 9381 medical records obtained from a reputed hospital in National Capital Region in India to build readmission predictive model.

A growing amount of literature is devoted to developing new tools for predicting readmission risks [8]. Numerous previous studies have analyzed the risk factors that predict readmission rates of patients with diabetes [9–16]. Jiang [9] studied demographic and socioeconomic factors which influence readmission rates. Eby [12] analyzed the readmission risk for a dataset of more than 52,000 patients in the Humedica network. Strack [13] studied the impact of HbA1c on

readmissions. Bhuvan et al. [16] evaluated different machine-learning algorithms considering both short-term and long-term readmissions for diabetic patients using public data of patients. The most common method used to predict readmission is stepwise multivariable logistic regression and the most common technique to evaluate the model performance is to use receiver operating characteristic (ROC) curve or C-statistic [17–19].

These models are difficult to implement in Indian healthcare scenario as little digitization of health data is available in Indian hospitals. Also, the use of International Classification of Diseases (ICD) codes to record primary and secondary diagnosis, and record of discharge disposition, admission source, and comorbidities are very rare. Thus, no comprehensive study could be found in the literature to investigate related research questions with respect to developing countries like India. Further, in contrast to any previous work, this study not only discovered risk factors that predict the risk of readmission but also identified individual as well as group of factors that are strong indicators of low risk of readmission along with the cost analysis using real-world data.

## Methods

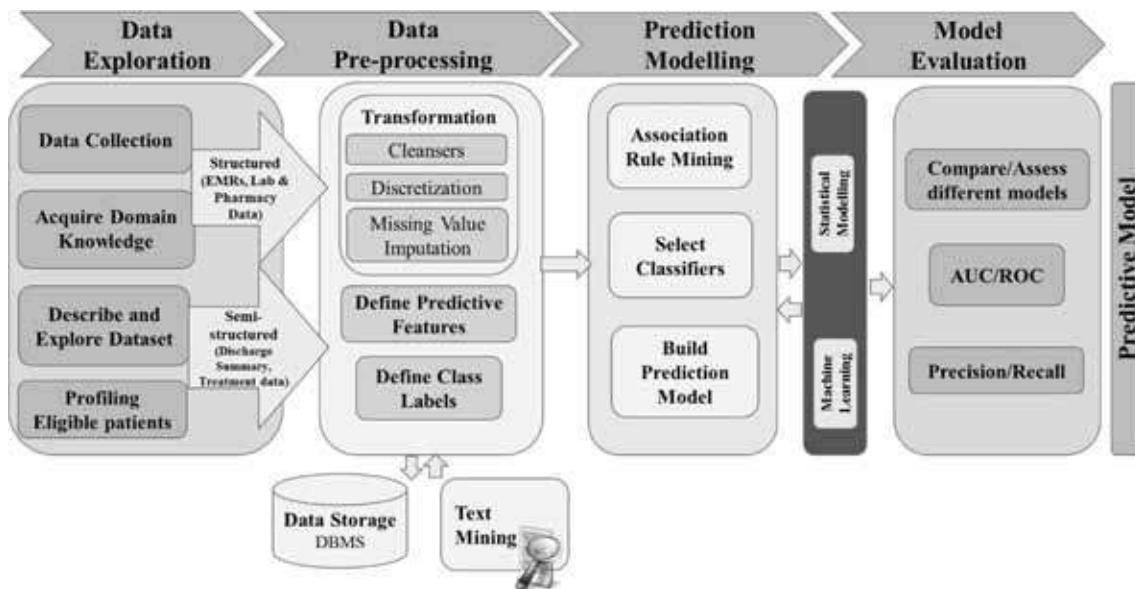
This section discusses the methodology to deal with the risk of readmission prediction problem for patients with diabetes. Figure 1 defines the major steps involved in building the predictive model.

### Data exploration

This phase explored the raw data to discover initial insights and recognize interesting actionable patterns. In this work, a dataset of 9381 diabetic patient encounters was extracted from their EMR system, over a period of 2 years (2013–2015) from an Indian hospital. It held approximately 7100 patients diagnosed with diabetes and servicing over 9300 hospital encounters. Before analyzing the data, all patient records and information were anonymized and de-identified. Continuity of patient encounters within the same health system was preserved. The necessary knowledge of the medical domain was acquired to investigate the dataset using valuable inputs from medical experts. The process of finding eligible dataset of patients with diabetes is depicted in Fig. 2.

### Data pre-processing

Medical data in the real world is noisy, inconsistent, and incomplete. So before building the prediction model, it is essential to pre-process the data efficiently and make it appropriate for predictive modelling. The impact of different pre-processing techniques on the classifier performance of logistic



**Fig. 1** Architecture of early readmission risk prediction modelling process

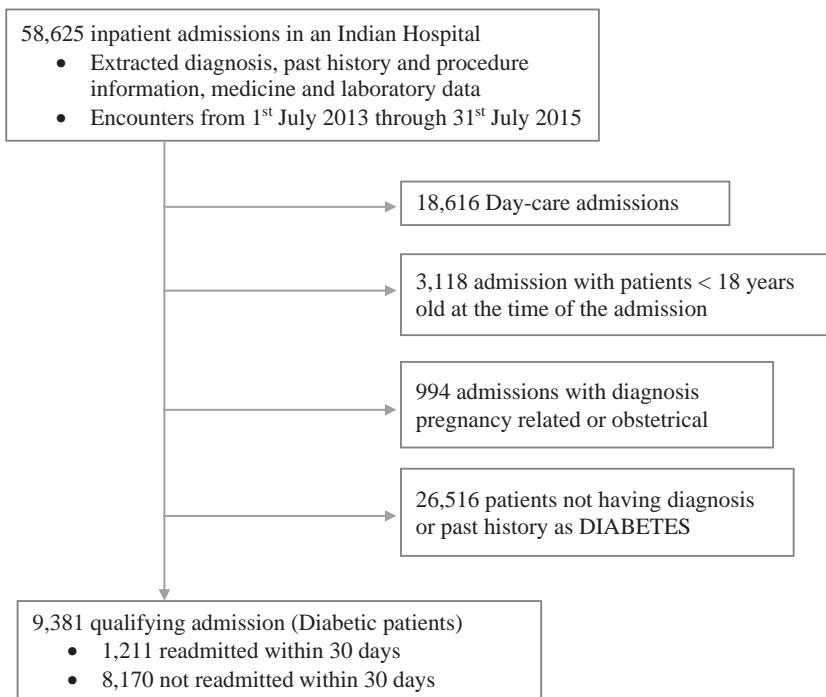
regression, naïve Bayes, and decision tree was assessed on various performance metrics such as area under curve, precision, recall, and accuracy by the authors [20]. Based on this study, it is concluded that selected data pre-processing techniques like feature selection, missing value imputation, and data balancing have a significant effect on hospital readmission predictive accuracy for patients with diabetes, with certain schemes proving inferior to competitive approaches. So feature selection and missing value imputation techniques

were implemented in this study to make the prediction more accurate.

#### *Data transformation*

Extreme, implausible values and outliers were removed to make data appropriate for training the predictive models. This study used mean/mode imputation (MMI) for imputing

**Fig. 2** Flow chart showing inclusion and exclusion of inpatient admissions in the analytic cohort



missing values [21]. Continuous vital signs and laboratory test result variables were classified based on standard medical criteria such as low, normal, and high categories. New variables were derived by considering the past history and longitudinal patterns. For example, the variable “Number of Inpatient visits last year” was defined as whether the patient had been hospitalized prior to the current encounter because it is highly associated with patients’ disease severity and potentiality to readmission. Data mining techniques like data cleaning, discretization (for numeric attributes like age, number of diagnoses, number of procedures), sorting, and aggregation were done to transform the data.

#### Define predictive features

Several brainstorming sessions with domain experts from a reputed Indian hospital were conducted. Through an extensive review of related studies [11–14, 22] and the help of domain experts, only attributes that are potentially associated with the diabetic condition or management influencing early readmissions were retained. Figure 3 lists the features which were used in building predictive models (*italic* features are derived features). The variables chosen for patient demographic and illness severity were gender, age, admission source, and time spent in the hospital. Many abnormal lab results and prescription drugs related to diabetes were also included to gage the acuity of patient’s condition.

#### Text mining

Unlike developed countries, use of ICD codes to record diagnosis is rare in Indian hospitals. In this study also, comorbidities were extracted from diagnosis and past history of the patient record which were free-form text using text mining techniques of database management system. Also, the number of procedures (surgeries, treatments, etc.) conducted on a patient in a hospital encounter was extracted from free-form clinical notes using text mining. The objective was to know the severity of patient’s condition. In this study, Mayo Clinic’s Clinical Text Analysis and Knowledge Extraction System (cTAKES) is being adopted [23]. It is an open-source natural language processing (NLP) system for extraction of information from clinical free text such as progress notes or a discharge summary. It can be used to create one or more pipelines to process clinical notes and to identify mentions of clinical named entities such as diseases and disorders, signs and symptoms, drugs and procedures in patient records. This study used SNOMED CT [24] dictionary included in the Unified Medical Language System (UMLS) [25] of cTAKES to extract number of procedures for a patient in a single encounter.

#### Defining class labels

The class labels (Yes or No) of the readmission attribute were determined by examining all patient records in the database to determine the first inpatient visit after discharge. Note that 30 days is chosen based on the criteria often used by government bodies in countries such as Canada, Australia, New Zealand, and the UK [1].

#### Predictive modelling

##### Classification

In this study, using five classification models (algorithms), namely naïve Bayesian, Logistic Regression, Random Forest, Adaboost, and Neural Networks classifier, a comprehensive set of experiments were performed to build prediction model using the tool WEKA 3.7. WEKA is a tool that provides machine learning algorithms to support data exploration, data mining, and prediction model development [26]. The reason of choosing these classification techniques is their wide-spread popularity and power to solve binary classification problems. Evaluation of all predictive models was done by using tenfold cross-validation procedure as this technique has low biasness and variance [27].

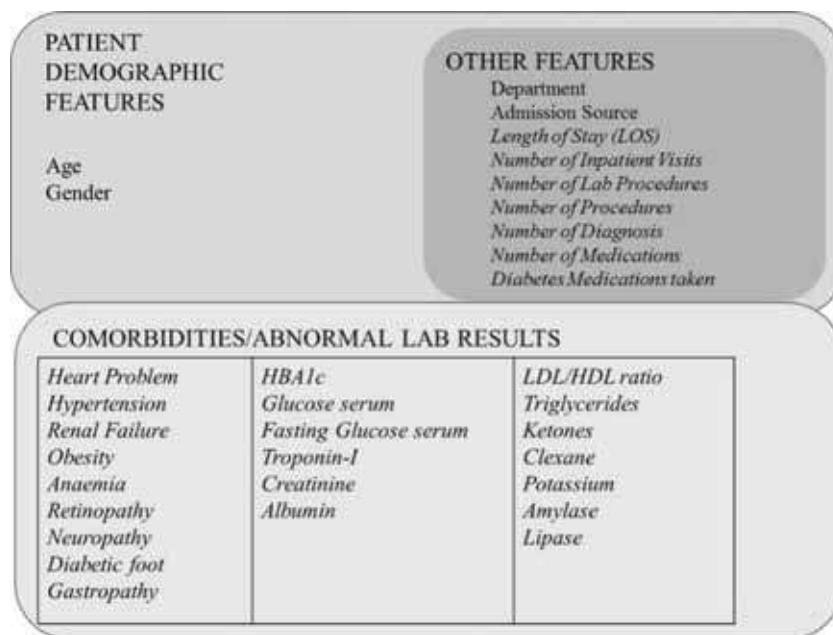
##### Feature analysis

There are two methods of carrying the feature analysis to discover critical risk factors [16]:

*Ablation study of risk factors*—an ablation study involves removing one factor at a time and comparing the accuracy of predicting readmission with this set of features with the accuracy obtained by considering all the features. Intuitively, removal of more important features should lead to decrease in accuracy. The difference in the evaluation measure is used as the estimate of the importance of the feature.

*Associative rule mining*—discovering the groups of factors that commonly occur among readmitted patients or those patients who were never readmitted can further enhance understanding about causes of readmission. Groups of consistently occurring factors that inference readmission rates can be revealed by associative rule mining using Apriori algorithm [28]. This study applied class-sensitive associative rule mining. Then for each rule, the number of patients was determined in the entire dataset that followed this rule. Within this set, the fraction of patients was computed which were readmitted and which were not. The rules with the highest fraction of readmissions indicate the factors that are strong predictors of high risk of readmission.

**Fig. 3** Features used in the risk of readmission prediction process



## Model evaluation

In this phase, various classification models were measured for quality or effectiveness using various evaluation metrics such as precision, recall, F-measure, ROC, and area under curve (AUC). Use of these metrics is popular in applications where data is skewed and where prediction of minority class is significantly important than the majority one. A system for predicting high-risk patients is only useful if a large fraction of patients at high-risk are correctly identified (i.e. high recall) without raising a large number of false alarms (i.e. high precision). ROC is ubiquitously known as one of the best metrics to evaluate classification models. The research paper by Davis [29] clearly proves a strong relationship between ROC space and precision-recall (PR) space. The paper also states that, though ROC curves are generally used to present results for dichotomous decision problems in machine learning when dealing with highly skewed datasets (like the dataset in this study), PR curves give a more useful picture of the performance of the classifiers. Therefore, PR curves were chosen as evaluation criteria in this study.

## Cost-sensitive analysis

Cost-sensitive analysis is a way to measure the potential savings of intervention on patients identified by the model as high risk. Let  $X$  be the cost incurred per readmission and  $Y$  be the cost per special diagnosis for patients predicted as Yes. Let the patient encounter instances of the dataset be defined according to the cost

matrix shown below. Cost matrix is one in which cost or penalty of classification will be specified for each element as in the confusion matrix.

$$\text{Cost Matrix} = \begin{bmatrix} \text{TP} & \text{FN} \\ \text{FP} & \text{TN} \end{bmatrix} \quad (1)$$

where TP, FP, FN, and TN correspond to true positives, false positives, false negatives, and true negatives, respectively.

*Without the prediction model:* All the instances where patients actually get readmitted (true positives and false negatives) incur a cost of  $X$ , hence defined by the below matrix:

$$\text{Cost Matrix without Prediction Model } (C_1) = \begin{bmatrix} X & X \\ 0 & 0 \end{bmatrix}. \quad (2)$$

*With the prediction model:* Considering that all patients who are predicted to get readmitted (true positives and false positives) would be examined with a special diagnosis which would cost  $Y$ , which in turn would prevent their readmission. The patient encounters who are predicted to be readmitted but who do not actually get readmitted would also incur a cost of  $Y$  for special diagnosis, which will just serve as a preventive measure in conservative hospital scenario. Hence, with the predictive model, cost matrix  $C_2$  is created as shown below. In this research, similar to the study done by Bhuvan et al. [16], special diagnosis is considered to be “one extra day” during the initial admission during which the doctor can conduct another diagnosis which might result in the discovery of new complications in the patient. Hence, the cost of special

diagnosis  $Y$  is considered to be the cost per 1 day of admission to the hospital.

$$\text{Cost Matrix with Prediction Model } (C_2) = \begin{bmatrix} Y & X \\ Y & 0 \end{bmatrix}. \quad (3)$$

The difference of cost matrix with and without prediction model gives the saved cost matrix as shown below.

$$\text{Cost Matrix of Savings } (S = C_1 - C_2) = \begin{bmatrix} X - Y & 0 \\ -Y & 0 \end{bmatrix}. \quad (4)$$

## Results

This section contains results consolidated from various experiments as described in the Methods section.

### Analysis of classifiers

Out of total 9381 records, 1211 (12.9 %) encounters were found as readmissions within 30 days of discharge from the hospitalization. Table 1 compares the different classification methods based on various evaluation measures.

Figure 4 compares the accuracy of different methods based on precision-recall curve. The dotted line shows the performance of a classifier at chance accuracy.

It is observed that best-performing algorithm for area under PR curve was Random Forest (0.296) followed by Logistic Regression (0.275). Neural Network (0.242) was a least impressive classifier in this case. Accuracy was high for all classifiers but ROC was low. The reason being, as the given data set is imbalanced, the classifier is biased towards majority class yielding a very optimistic accuracy estimate.

### Identifying the critical risk factors

#### Using ablation study

As Random Forest was found as the most accurate classifier, it was used to identify the importance of each risk factor in

identifying high-risk patients using an ablation study. The difference in the AUPRC was used as the estimate of the importance of the feature. Figure 5 shows the evaluation score (i.e. decrease in AUPRC) for each factor. It was observed that department, the number of inpatient visits, and length of stay (LOS) were most important for identifying high-risk patients. It means that removing department attribute results in the biggest drop in AUPRC, and removing number of lab procedure attribute results in the biggest gain in AUPRC. However, the impact on each case was fairly small. Furthermore, it was observed that the high percentage of readmissions happened in Oncology, Renal, and Liver Transplant departments. Clinicians need to do detailed investigation of root causes in these departments and implement adequate interventions to lower the risk of readmission.

#### Association rule interpretation

Prominent association rules have been given in Table 2 with respective support and confidence.

Rule 1 indicated that diabetic patients with no past history of inpatient visits in the last 1 year, creatinine test normal, no renal, gastropathy, or diabetic foot problem, no ketones detected, and potassium levels normal in their blood work were less probable (4.98 %) to be readmitted within 30 days, as only 63 such patients out of 1264 were readmitted within 30 days in the dataset. Similarly, rule 4 indicates that patients admitted through emergency and having hypertension were more probable (14.59 %) to get readmitted within a month.

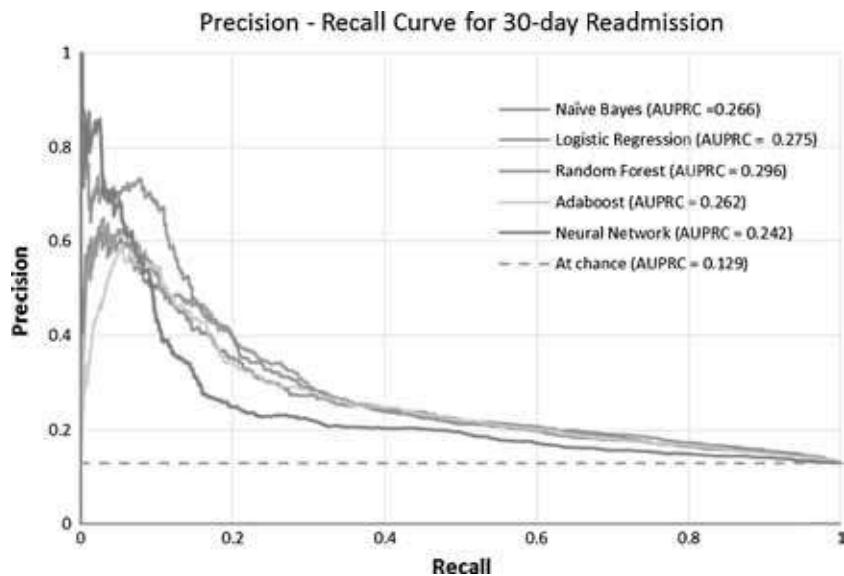
### Cost analysis

By using the actual cost of readmissions, cost analysis was conducted to calculate the possible savings if the model is implemented. The average cost of readmission of patients with diabetes was calculated from the data provided by an Indian hospital. The data specified that cost of readmission of patients with diabetes was INR 137,304,521 for 1078 total readmissions (data was not available for few readmissions). Hence, cost per readmission approximately equaled to INR 127,370 (INR 137,304,521/1078). The cost of special

**Table 1** Comparison of different classifiers in predicting 30-day readmission for patients with diabetes

Classifier	Precision	Recall	F-measure	Accuracy	Area under ROC (AUC)	Area under PR curve (AUPRC)
Naïve Bayes	0.303	0.249	0.274	82.90 %	0.678	0.266
Logical Regression	0.575	0.085	0.148	87.39 %	0.674	0.275
Random Forest	0.717	0.067	0.122	87.61 %	0.688	0.296
Adaboost	0.548	0.099	0.168	87.31 %	0.675	0.262
Neural Network	0.583	0.073	0.129	87.36 %	0.624	0.242

**Fig. 4** Comparison of the accuracy of different classifiers based on precision-recall curve

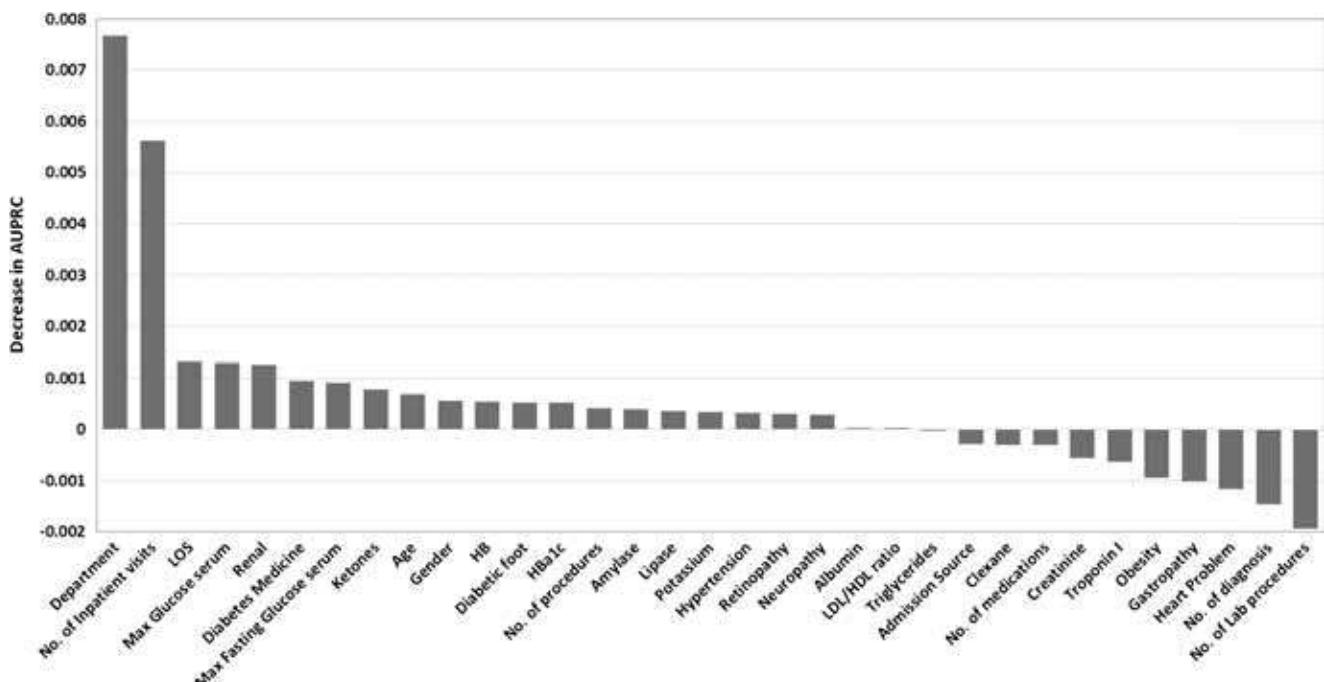


diagnosis was considered to be cost per 1 day of admission to the hospital. From the dataset, it has been found that the average time in the hospital across diabetic patient encounters was 5.633 days. So, the cost for 1-day admission was considered to be INR 22,611 (INR 127,370/5.633). Therefore, the values of  $X$  and  $Y$  were INR 127,370 and INR 22,611 respectively. Hence, the saved cost matrix was considered as follows:

$$\text{Cost Matrix of Savings } (S) = \begin{bmatrix} X - Y & 0 \\ -Y & 0 \end{bmatrix} = \begin{bmatrix} 104759 & 0 \\ -22611 & 0 \end{bmatrix}.$$

The costs saved by different models using this cost matrix are given in Table 3.

It has been observed that the naïve Bayes model would save the maximum cost of INR 15.92 million for a total number of 9381 diabetic patient encounter instances. The reason being it had the maximum number of true positives (302) in comparison with Random Forest true positives (81). Also, a large saving amount of INR 104,759 for each true positive outweighed the high number of false positives (997) of naïve Bayes. High F-measure (0.274) of naïve Bayes was also



**Fig. 5** Importance of individual risk factors for identifying high-risk patients for readmission

**Table 2** Prominent association rules

No.	Readmission status Total number of instances Association rules (consequents)	Yes 1211	No 8170	Total
				Class-wise matches (in %)
1	inpatient_visits_last_year = 0; Creatinine = Normal; Renal = NO; diabetic_foot = NO; Ketones = None; Gastropathy = NO; Potassium = Normal	4.98	95.02	1264
2	Gender = Female; inpatient_visits_last_year = 0; Renal = NO; Diabetes_med = YES; Retinopathy = NO; diabetic_foot = NO; Gastropathy = NO; Obesity = NO; Potassium = Normal	5.88	94.12	1020
3	Gender = Male; Hemoglobin = Normal; Creatinine = Normal; Renal = NO; Retinopathy = NO; Neuropathy = NO; diabetic_foot = NO; Heart_problem = YES; Gastropathy = NO	6.00	94.00	1000
4	Admission_source = EMERGENCY; Hypertension = YES	14.59	85.41	2803
5	Hemoglobin = <11; Albumin = <3.5	16.75	83.25	2418
6	Creatinine = > 1.4; Hemoglobin = <11	16.78	83.22	2396

an indicator of the same. Adaboost was the second best classifier with INR 10.72 million savings.

## Discussion

As per precision-recall curve, random forests emerged as the best algorithm. These results are in sync with various existing studies done before. Bhuvan et al. [16] found random forest as the best classifier for readmission prediction using US public health data. Caruana [30] had done an empirical comparison of supervised learning algorithms using various datasets. Their study showed that boosted trees, random forests, and bagged trees performed the best in most of the cases. The results of other diseases also showed best results when random forests were used [31]. During ablation study, the most critical risk factors were identified as the department of admission, the number of inpatient visits, and LOS. Bhuvan et al. [16] discovered the similar risk factor i.e. the number of inpatient visits as a critical one. They also observed discharge disposition and admission type as most important risk factors for identifying high-risk patients. As the Indian hospital under study does not collect discharge disposition information, this factor was not considered in this study though, and admission

type (admission source in this study) did not emerge as an important factor.

This study used Apriori algorithm to retrieve several prominent association rules. When the prediction model predicts that this particular patient will not get readmitted, as a medical practitioner, one can use this matched rule to statistically understand that similar patients (who had similar diagnosis) were not readmitted in the past. It might help him to get him more insights and would assist him in further diagnosis. He would understand that such patients are less prone to risk. Such interpretations from all the rules could be very useful for taking informed decisions and gaging the risk factor. It has been observed that most of these rules can only help to decide about the probability of patients not getting readmitted within 30 days, due to the biased distribution of the dataset.

Cost analysis, by using the actual cost of readmissions, is used to calculate the possible savings if the model is implemented. Models with different machine learning algorithms are tuned to maximize the cost saved by selecting appropriate parameters. It has been observed that the naïve Bayes model would save the maximum cost of INR 15.92 million for a total number of 9381 diabetic patient encounter instances. Similar cost savings were reported by Bhuvan et al. [16] also. For developing countries like India where a large number of patients admit to a hospital, this is a huge saving.

The work done in this paper is the first one generating prediction model to access the risk of early readmission for patients with diabetes especially by using real-world data from an Indian hospital. Consequently, results are more reflective of the problem of readmissions among patients with diabetes in Indian context. Also, this model never suggests healthcare personnel to give less attention to those patients predicted not to be readmitted, but prompts extra attention to those who are predicted to get readmitted. In this sense, the designed model is conservative in nature and is safe to use in healthcare institutions as it enhances preventive strategy along with

**Table 3** Comparison of cost saved by different machine learning models

Classifier	Cost saved for 9381 patients (INR)
Naïve Bayes	15,922,573
Logical Regression	9,071,741
Random Forest	7,761,927
Adaboost	10,332,591
Neural Network	7,794,299

saving cost associated with readmission. Moreover, those patients who are predicted to be readmitted would receive special diagnosis at an earlier stage which might save a lot of lives. Hence, this study concludes that the model could be incorporated in healthcare institutions to witness its effectiveness. This research considers the hypothesis that the special diagnosis would prevent the actual readmission. It considers the cost of special diagnosis to be the cost per 1 day of admission where the doctor can run another diagnosis to discover possible complications. Though these hypotheses seem legitimate, they need to be tested by incorporating the research model in real healthcare systems.

## Conclusion and future work

Predicting hospital readmission rates can increase the efficiency of initial treatment at hospitals which can save a lot of lives. In this research study, the real-world hospital data of inpatients having diabetes as an existing condition in conjunction with other medical illnesses is analyzed. The objective was to build a predictive model to identify patients who have a higher likelihood of being readmitted. This study classifies the patients into two different risk groups of readmission (Yes or No) within 30 days of discharge based on patients' characteristics using 2-year clinical and administrative data (~10,000 encounters and ~30 attributes) from an Indian hospital. It then evaluates the classification accuracy using five different data mining classifiers—naïve Bayesian, Logistic Regression, Random Forest, Adaboost, and Neural Networks, and the best classifier is chosen. It proposes an architecture of this prediction model and then explain each step in detail. Some of the key factors that drove readmission are the department of admission, number of times a patient is formerly admitted as an inpatient, and length of stay. Random forest is found to be the optimal classifier for this task using the evaluation metric area under precision-recall curve. By mining hidden patterns in the diagnosis, medications, lab test results, and basic characteristics of patient related to readmissions, this model finds a strong set of statistically significant implications or association rules. A ranked list of such rules can be instrumental for a doctor prior to diagnosis. As an additional safety check, the doctor can verify the prevalence (or lack of it) of these conditions to every patient, increasing the effectiveness of diagnosis and better medical decisions. From the cost analysis, it is observed that a cost of INR 15.92 million can be saved for 9381 instances of diabetic patient encounters. Saving such huge amount cost is significant for healthcare system.

This research targets diabetic patients only. Such analysis needs to be carried for other top health conditions like heart disease and schizophrenia in Indian healthcare. In the future studies, planned and unplanned (emergency) readmissions need to be considered. Several critical features in the medical

records, like family history (to find hereditary information), lifestyle, and social factors, need to be collected. The conversation between doctor and patient can also be collected which might help to extract essential features corresponding to patients' willpower and attitude by text mining techniques which in turn might improve the intelligent models to identify patients at high risk of readmission.

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## Compliance with ethical standards

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

# A prospective observational study to assess the effectiveness of an electronic health (E-health) and mobile health (M-health) platform versus conventional care for the management of diabetes mellitus

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**Abstract** We undertook a prospective observational study to evaluate the effectiveness of an electronic health (E-health)/mobile health (M-health) platform versus conventional care for the management of diabetes mellitus. One hundred nine patients with type 2 diabetes were recruited from a tertiary care hospital in India. Thirty-nine patients were enrolled in the Diabetes Care 24x7® (DC) arm, whilst 70 were enrolled in the control (conventional care) group. Primary endpoints included fasting blood sugars (FBS), post-prandial blood sugars (2hr PP) and HbA1c level. Secondary endpoints included knowledge of the disease and its impact on quality of life. Outcome measures were assessed at the initial and fifth month visit. Males/females ≥18 years, attending the diabetes clinic, met the inclusion criteria. Pregnant women were excluded. Eighty six patients completed the study (33 in DC and 53 in the control group). A statistically significant reduction was observed in HbA1c ( $8.8 \pm 1.2$  to  $7.4 \pm 1.3$ ,  $p$  value = 0.001). FBS/2hr PP blood sugars showed a trend towards improvement in both arms, but intergroup correlation at study end did not reach statistical significance ( $p$  value = 0.5). Diabetes knowledge scores ( $19.9 \pm 2.5$  vs.  $17.9 \pm 3.98$ ,  $p$  value = 0.005) and quality of life indices ( $88.5 \pm 7.8$  vs.  $83.5 \pm 10.7$ ,  $p$  value = 0.015) showed a statistically significant

improvement in the intervention arm. A multifaceted E-health/M-health platform has great potential to reach patients in remote corners of the globe and improve not only their disease parameters but their knowledge scores and quality of life indices. Further longitudinal studies are needed to assess the true potential of this technology to impact diabetes management in the twenty-first century.

**Keywords** Telemedicine · Type 2 diabetes · HbA1c

## Introduction

According to the International Diabetes Federation (IDF), diabetes mellitus (DM) claims a life every 6 s. Globally, 415 million individuals are currently affected and another 46.5 % potentially undiagnosed. Seventy-five percent of this population subset resides in low-middle income countries, where resources may be scarce and expenditure on health care somewhat limited. India has over 66 million documented individuals with DM [1]. Unfortunately, awareness of the disease and knowledge of guidelines do not necessarily translate into optimal clinical practice and follow-up. In 2006, a cross-sectional survey of 819 participants with type 2 diabetes mellitus (T2DM), from over 20,000 houses, revealed that only 13 % had a glycosylated haemoglobin (HbA1c) done within the past year. A mere 16.2 % had a dilated eye exam as per standard of care; only 32.1 % had their lipid profile checked; and approximately 63 % had a blood pressure >140/90 mmHg [2].

In an attempt to fill this void in practice, the past few years have seen an emergence of various electronic/web-based healthcare delivery applications in the Indian market. It is well

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documented that the use of an electronic medical record (EMR) can help improve diabetes care in an evidence-based fashion, reduce medical errors and even result in savings on healthcare expenditure. Interactive online sites function to increase knowledge, self-engagement and efficacy; enable behaviour change; and encourage dialogue between physicians and patients [3].

Previous studies globally have demonstrated a reduction in morbidity and mortality, when supplementing or delivering diabetes care via a web-based programme [3]. An EMR can certainly improve care delivery in an evidence-based fashion [4]. Outcome trials ranging from a duration of anywhere between 12 and 120 weeks using websites have shown a HbA1c reduction of anywhere between 1.2 and 16 %. Videoconferencing itself has increased patient satisfaction. The utilization of an interactive voice response (IVR) system successfully decreased healthcare utilization costs amongst elderly patients with diabetes, in the Veterans Affairs (VA) hospital system. This interactive modality resulted in a drop in emergency room visits and a 16 % increase in outpatient clinic visits [3]. Haddad et al. further analysed the use of cell phones and short message services (SMS) to deliver diabetes care in a resource-constrained environment. An increase in knowledge scores and a statistically significant improvement in glycemic control was seen [5]. In developing countries, where healthcare delivery in remote areas is lacking [6], whilst remote monitoring in DM does offer hope, a recent systematic review incorporating studies across seven countries concluded that the optimal design of a telemedicine system is still unclear [7]. Tools such as cell phones would enable doctors to bring medicine to the community.

In 2014, the Division of Endocrinology at the Max Super Speciality Hospital, Saket, in conjunction with mCURA Mobile Health Private Limited, designed an E-health and M-health platform (Diabetes Care 24x7<sup>®</sup>) to better serve the needs of our patients. The system enables both domestic and international patients with diabetes to connect with their healthcare provider from any corner of the globe. Patients have an option of either calling, using modalities such as Skype<sup>®</sup> or FaceTime<sup>®</sup>, or sending an email with any queries that they may have. A team of trained diabetes educators is available 24 h a day, 7 days a week, to respond in a timely fashion. Issues beyond the scope of education, diet advice or a medication query are subsequently escalated to a doctor on call/consultant.

All patient data is recorded online, with the user interface giving the healthcare provider a complete overview of the patient's past history, visit encounters, active issues, ongoing medication use, glycemic control, etc. The data itself is stored on a secure hospital server. Any changes in management are documented. To close the loop, the patient/next-of-kin also gets a notification of the advice given via either email or a text message. Lastly, evidence-based guidelines are built into the

software algorithm. For example, if a patient has not had an HbA1c in the past 3 months or missed a follow-up appointment, the system will flag their file for the attention of the healthcare provider and send a simultaneous alert to the patient. Educational videos, emails and daily tips also ensure that awareness about diabetes and its complications is maintained. This ensures that patients remain engaged in their health and limits the number of patients that are lost to follow up. The programme is unique, as aside from serving as an EMR, it empowers patients through education whilst providing them multiple avenues to be in constant contact with their providers.

To the best of our knowledge, there has been no study looking at the effectiveness of such a multifaceted programme in India, in terms of both improving patient care and assessing its impact on patient knowledge and quality of life. The objective of this pilot study was to assess the impact of Diabetes Care 24x7<sup>®</sup> on these very parameters in patients with T2DM.

## Subjects and methods

This prospective intervention study was designed to assess the effectiveness of an E-health and M-health platform (Diabetes Care 24x7<sup>®</sup>) versus conventional care for management of diabetes in a cohort of Indian patients with T2DM. The primary endpoints included tangible markers of improved glycemic control: fasting blood sugars (FBS), post-prandial blood sugars (2hr PP) and HbA1c level. The 2hr PP levels were chosen as our traditional Asian-Indian diets are carbohydrate-rich and lead to significant post-prandial glycemic excursions [8]. Secondary endpoints included assessing a patient's knowledge of their disease and the impact of diabetes on their quality of life.

## Subjects and setting

All males and females above the age of 18 who attended the diabetes clinic of Max Super Speciality Hospital, Saket, New Delhi, and agreed to participate in our Diabetes Care 24x7 programme met the inclusion criteria for the Diabetes Care 24x7 (DC) cohort. The rest of the patients were eligible for the control group. We excluded pregnant women with T2DM and those who did not agree for follow-up. The study received approval from both the Scientific and Ethics committee of Max Hospital and was carried out from October 2014 to May 2015.

## Control group

The control group saw their physician and received diabetes education/counselling as per routine practice, only at their scheduled visits.

## Intervention/DC group

Aside from their scheduled visits with an endocrinologist, who practised as per norm, patients in the DC cohort had a weekly telephonic follow-up by team physicians/diabetes educators to assess their glycemic control and trouble shoot any issues. They also were able to access educational videos and received daily tips on managing their disease, via email or text message.

Biochemical parameters of all patients were recorded both on their initial and fifth month follow-up visits. Knowledge of DM and its impact on quality of life were measured using standardized assessment tools such as a modified Diabetes Knowledge Test (DKT) and Quality of Life (QOL) questionnaire [9–11]. The questionnaires were filled by all participants on their initial and fifth month follow-up visits. Lastly, as an outcome marker for safety, we qualitatively documented if patients had had an episode of hypoglycaemia in each group by using a simple YES or NO answer format. Hypoglycaemia was defined as a blood sugar  $\leq 70$  mg/dL ( $\leq 3.9$  mmol/L) [12].

## Statistical analysis

The statistical analysis was done by way of computing mean and standard deviation of all variables in the control and DC groups. For qualitative variables such as smoking and FBS, contingency tables were prepared. Improvement of DKT, QOL, FBS, 2hr PP and HbA1c from pre to post levels was tested by a paired *t* test, done separately in the DC and control groups. For comparison of the DC and control groups, these improvements were compared with student *t* test. Improvement in DKT and QOL in males and females was initially compared by using a paired *t* test for each score. Presence of hypoglycaemia in both the groups was assessed by chi-squared test. All tests were done at a 5 % significance level using SPSS 20.

## Results

One hundred patients with diabetes were randomly screened for the control group, and out of which, 70 patients were enrolled. Baseline demographic data is given in Table 1, and a graphical version of the primary/secondary outcome data is presented in Figs. 1 and 2. At the end of the study, 17 were lost to follow up, leaving 53 for the final analysis. For the Diabetes Care 24x7® (DC) cohort, 80 patients were screened, out of which 44 agreed to participate and 5 were excluded due to logistical difficulties. Out of the remaining 39 patients, 6 were lost to follow up, leaving 33 patients for the final analysis.

In each arm, 74.3 % of the participants were males, with the entire study group having a mean age of  $52.6 \pm 10.7$  years. All of the patients had T2DM, with the average body mass index

(BMI)  $27.9 \pm 4.5$  kg/m<sup>2</sup> in the control group and  $27.6 \pm 6.0$  kg/m<sup>2</sup> in the DC group. At baseline, the DC subset had a higher FBS ( $177.3 \pm 74.3$  mg/dL), a higher 2hr PP ( $242.2 \pm 96.0$  mg/dL) and a higher baseline HbA1c ( $8.8 \pm 1.8$  %), *p* values 0.023, 0.17 and 0.022, respectively

Within the DC group itself (Fig. 1), HbA1c improved from  $8.8 \pm 1.8$  % to  $7.4 \pm 1.3$  %, *p* value = 0.001, versus within the control group ( $7.9 \pm 1.8$  % to  $7.4 \pm 1.7$  %; *p* value = 0.455), post intervention. However, FBS levels in the DC group improved more than the control arm ( $119 \pm 21.5$  mg/dL vs.  $122.7 \pm 51.1$  mg/dL; *p* value 0.7) and within the DC group as well; the change from baseline was highly significant ( $177.3 \pm 74.3$  mg/dL to  $119 \pm 21.5$  mg/dL, *p* value = 0.000). Interestingly, when using the control group as its own benchmark, the reduction in FBS levels at the end of the study was also highly significant ( $134.8 \pm 65.8$  mg/dL to  $87.6 \pm 70.5$  mg/dL; *p* value = 0.000). A statistically significant improvement in 2hr PP levels was also seen in both arms of the study, when each group was used as its own comparison (DC group ( $242.2 \pm 96.0$  mg/dL to  $165.8 \pm 42.3$  mg/dL; *p* value = 0.000), control group ( $216.3 \pm 81.4$  mg/dL to  $174.1 \pm 2.5$  mg/dL; *p* value = 0.000)). Of note, even though the DC group had lower 2hr PP levels, a comparison with the control cohort did not yield statistical significance ( $165.8 \pm 42.3$  mg/dL vs.  $174.1 \pm 72.5$  mg/dL; *p* value = 0.5). Lastly, it was noted that 15.3 % of the DC cohort answered “yes” with regard to having an episode of documented hypoglycaemia, versus 18.57 % of the control group.

In terms of the secondary outcomes, it was noted that the DKT score (Fig. 2) was higher at baseline in the DC group versus the control group ( $17.1 \pm 2.9$  vs.  $15.9 \pm 3.8$ , *p* value = 0.07). This was still reflected in the scores post intervention, with again a greater improvement seen in the DC cohort ( $19.9 \pm 2.5$  vs.  $17.9 \pm 3.9$ , *p* value = 0.005). Quality of life indices (Fig. 2) were also higher in the DC group at baseline ( $77.7 \pm 11.9$  vs.  $71.7 \pm 14.1$ , *p* value = 0.02). Again, post study, the DC group showed the greatest and statistically significant improvement ( $88.5 \pm 7.8$  vs.  $83.5 \pm 10.7$ , *p* value = 0.015). When assessing the entire study population as a whole, knowledge scores and quality of life indices improved equally in both males ( $16.3 \pm 3.4$  to  $18.7 \pm 3.5$  and  $73.8 \pm 14.7$  to  $85.4 \pm 9.6$ , respectively) and females ( $16.3 \pm 3.9$  to  $18.7 \pm 4.1$  and  $73.8 \pm 11.2$  to  $85.9 \pm 10.9$ , respectively).

## Conclusions

In this prospective interventional study of adults with T2DM, the efficacy of an E-health/M-health platform (Diabetes Care 24x7®) was assessed in terms of improving glycemic control, patient knowledge and quality of life scores. There was a statistically significant improvement in tangible parameters like HbA1c in the intervention group, with a qualitative lower

**Table 1** Demographic and clinical characteristics of sample population ( $n = 109$ )

Parameters	Control ( $n = 70$ ) (Mean $\pm$ SD)	DC ( $n = 39$ ) (Mean $\pm$ SD)	Total ( $n = 109$ ) (Mean $\pm$ SD)
Age (years)	51.15 $\pm$ 10.39	53.4 $\pm$ 11.07	52.55 $\pm$ 10.65
Gender			
Males	52 (74.3 %)	29 (74.3 %)	$p$ value = 0.993
Females	18 (25.7 %)	10 (23.1 %)	$p$ value = 0.993
Occupation			
Full-time	47 (67.1 %)	30 (76.9 %)	$p$ value = 0.282
Others (retired, housewives, students)	23 (32.9 %)	9 (12.9 %)	$p$ value = 0.282
Height (cm)	164.64 $\pm$ 13.60	161.42 $\pm$ 10.58	163.99 $\pm$ 13.02
Weight (Kg)	75.88 $\pm$ 15.73	76.5 $\pm$ 20.93	75.19 $\pm$ 17.77
BMI (Kg/cm <sup>2</sup> )	27.97 $\pm$ 4.47	27.62 $\pm$ 6.01	$p$ value = 0.807
Pulse (beats/min)	84.19 $\pm$ 21.87	80.66 $\pm$ 14.55	83.67 $\pm$ 20.85
B.P:S (mmHg)	120.19 $\pm$ 19.46	125.26 $\pm$ 16.59	122.33 $\pm$ 18.35
B.P:D (mmHg)	72.21 $\pm$ 10.72	73.14 $\pm$ 9.79	4.4 $\pm$ 8.37
FBS (mg/dL)	143 $\pm$ 58.33	177.32 $\pm$ 74.26	$p$ value = 0.023
PP (mg/dL)	216.28 $\pm$ 81.42	242.18 $\pm$ 96.04	$p$ value = 0.17
Baseline HbA1c	7.88 $\pm$ 1.82	8.80 $\pm$ 1.81	$p$ value = 0.022
Cholesterol (mmol/L)	159.20 $\pm$ 44.75	159 $\pm$ 56.35	159.12 $\pm$ 46.68
Triglycerides (mmol/L)	226.51 $\pm$ 325.05	162.62 $\pm$ 142.87	199.13 $\pm$ 262.60
HDL (mmol/L)	37.54 $\pm$ 8.04	39.66 $\pm$ 9.03	38.46 $\pm$ 8.46
LDL (mmol/L)	88.29 $\pm$ 27.00	88.74 $\pm$ 37.43	88.49 $\pm$ 31.8
Smokers	10 (14.3 %)	2 (5.1 %)	—
Alcohol intake	10 (14.3 %)	6 (15.4 %)	—

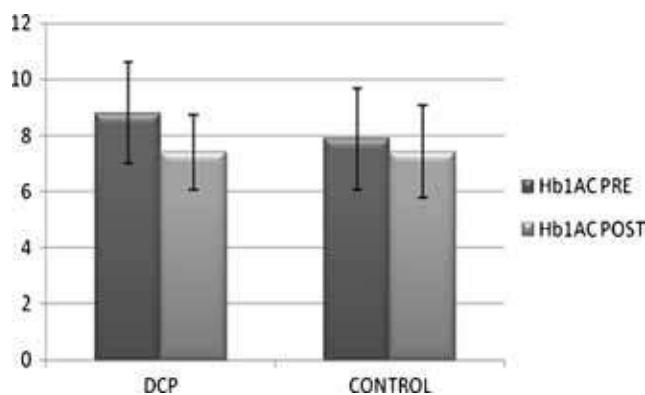
documentation of hypoglycaemia. Knowledge and QOL scores seemed to improve as well. Interestingly, both groups showed a decrease in FBS and 2hr PP values, with no statistical significance between the two arms of the study. Having said this, there was a greater trend towards improvement in the DC group overall.

This study adds to the literature in several ways. Firstly, to the best of our knowledge, Diabetes Care 24x7® is the first to integrate all the aforementioned modalities of healthcare delivery into one system and demonstrate feasibility of use in a resource limited environment. Again, aside from serving as an EMR, the system hosts an interactive educational website; allows patients, both national and international, to interact with their physicians using SMS, IVR, emails and videoconferencing; promotes education through daily alerts; and gives backend support 24 h a day. A trend towards better glycemic

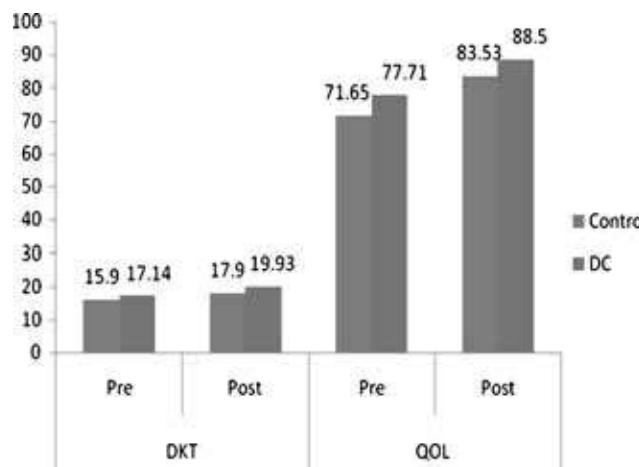
control, lower HbA1c, lower hypoglycaemia, increased patient education and improved patient satisfaction is evident in the study group. This is in keeping with historical data where aforementioned facets of this platform have been studied in an individual fashion.

The role of self-care in the management of any illness, let alone DM, is crucial to achieving long-term success and treatment satisfaction. It is well documented that individuals with diabetes can make a tremendous impact on the development/progression of their disease by taking an active role in the management of their illness [13]. A systemic review by Heinrich et al. further reinforces the fact that self-monitoring of blood glucose, knowledge of disease and diabetes-specific QOL scores improve with an intervention that aids in self management [14]. By fostering ongoing dialogue and continuous interaction, we feel that the Diabetes Care 24x7® platform further serves to motivate and engage patients in the management of their diabetes. This is reflected by the improved knowledge and QOL scores in the intervention arm.

We have several limitations in our study. The platform is new and continuously being upgraded based on patient and provider feedback. Due to this being a pilot project, we were not able to recruit large numbers of patients and lost some to follow up. We were not able to truly randomize the patients as the enrolment was dependent on patient choice. Again, patient education/socioeconomic status (greater number of full-time employees in the intervention arm) and motivation may have played a part in them choosing the intervention arm. It was also not possible to blind the investigators given the interactive nature of the intervention, creating the possibility of some bias when providing education. As with any intervention



**Fig. 1** Change in HbA1c levels (%) in both the intervention and control groups



**Fig. 2** Comparison of Diabetes Knowledge Test scores and QOL scores between the intervention (DC) and control groups

involving technology, there will be logistical difficulties. We were not able to recruit some patients as they lived in very remote parts of the country and had limited connectivity. Due to this being an initial project, we did not include our international patients (e.g. those from Afghanistan, Middle East and Africa) in the treatment cohort. We hope to do so in a larger prospective study.

Due to the short duration of the study, only a few markers of disease control were measured. We are yet to understand the impact of such an intervention on other entities such as blood pressure and lipid parameters, development of microvascular complications and follow-up rates. A partial explanation for the difference seen in the FBS and 2hr PP values is that a FBS and corresponding 2hr PP values are not comprehensive markers for glycemic control. These solitary values were done in a lab and could reflect study bias if patients adhered to their medications/lifestyle modification the day prior to getting the blood test done. Due to the cost of testing, patients often do not invest in a glucometer/test strips and rely on HbA1c/sporadic lab values to assess their disease control. Furthermore, the patients lost to follow up may have skewed the data in favour of a null hypothesis. As of yet, we do not have data on the economic viability of this project and the potential cost savings that it may lead to. DKT and QOL scores could have been higher at baseline in the intervention arm as this group may possibly be more motivated about their disease management. Hence, they chose to participate in the programme. Interestingly, standard of care in the control arm also involves the diabetes educators checking up on patients post visit, albeit not as frequently as the intervention group. This may have led to a lesser difference in the DKT and QOL scores, between the two groups. Again, larger studies are needed to truly elucidate the impact of this intervention on knowledge and quality of life.

In summary, the world is heading towards the digital age, and technology is touching the remote corners of the globe. The need of the hour is to develop better cost-effective wide-

ranging healthcare delivery platforms that decrease morbidity and mortality, whilst empowering patients to take control of their disease process and in turn improve patient satisfaction/quality of life. Ultimately, further longitudinal studies are needed to assess the true impact of programmes like Diabetes Care 24x7®, to ensure that we are providing the best possible patient-centric care and bringing medicine to the doorstep of the community.

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**Compliance with ethical standards** This study protocol, CRF and informed consent form were approved by the Institutional Ethics Committee of Max Super Speciality Hospital (a Unit of Devki Devi Foundation) prior to initiation of the study. Proper informed consent was taken from each and every study participant.

**Conflict of interest** The authors declare that they have no competing interests.

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2. Events: Latest events with the venue and dates
3. Newsletter: Latest information for diabetes educators
4. Journal of Diabetes Education Quarterly journal is uploaded
5. News and Announcements:
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### BOOK REVIEW

REVIEW OF 3RD EDITION OF RSSDI TEXT BOOK OF DIABETES MELLITUS : Editor-in-Chief – Hemraj B Chandalia, Executive Editor – Gumpenay Ramachandra Sridhar, Editors – Ashok Kumar Das, Sri Venkata Madhu, Viswanathan Mohan, Paturi Vishnupriya Rao

The third edition of RSSDI text book contains contributions from those who have been practicing / teaching Diabetology for many years, similarly the editors too. Most of the contributors are from within the country with many years of experience behind them. A few non resident Indians have made useful contribution. This edition as pointed out by editor – in – chief has gone on considerable revision from the first two editions. This only shows the importance of making an attempt to have our own text books and keep revising, based on the experience.

You name any thing in diabetes, this book has it. A few topics which are generally not paid much attention – like complexity of Insulin resistance, the criteria applicable in our country for metabolic syndrome, care of elderly diabetic, musculo-skeletal manifestation in diabetes are well covered. Malnutrition modulated Diabetes Mellitus and late onset of auto immune diabetes (LADA) as seen in our country is dealt with in detail.

The flow chart on management of diabetic keto-acidosis is useful and should be available in all ICU (Intensive Care Unit). The colour pictures of retinopathy and foot lesions are well presented. The usefulness of alternate therapy available in the country is extensively discussed. The Appendix is retained and gives a lot of information applicable to Indian subjects like BMI, waist circumference and laboratory values both in SI units and conventional units. The Index has reached perfection. Some controversial issues are mentioned in individual chapters but I wish an exclusive chapter was dedicated to controversies like classification of LADA and early use of insulin in these patients, need for revising the diagnostic plasma glucose values both in non-pregnant and pregnant diabetic, use of insulin in Type 2 diabetes at the time of detection to overcome the gluco and lipo-toxicity, safety of use of long acting insulin analogs during pregnancy, use of human insulin vis-à-vis insulin analogs, safety of DPP4 inhibitors, SGLT2 inhibitors and other new oral hypoglycemic molecules. Use of quantity and type of fat in the diet, role of low glycemic index diet in the management of diabetes etc.,

This book is a must for anybody who practices and teaches diabetes and students. The availability of this excellent text book has made western text books irrelevant to our country. The Novel feature of this book is mentioning the chapter number on the right edge of each page but single volume covering so many topics is bulky and heavy. I wish it was brought out in two volumes.

**Prof. (DR.) C. MUNICHOODAPPA**

Bengaluru

## Announcements for Research Grant

- For providing research grants, RSSDI invites proposals from Indian scientists, interested in conducting original research in the field of diabetes mellitus. Furthermore, limited grants are also available for the students of medical colleges for smaller projects.
- There is no deadline for submission of the proposals, which can be sent throughout the year. These proposals may fall into one of the following three categories:
  1. Projects involving funding up to Rs 40,000 per project (preference will be given to young scientists <40 years).
  2. Projects involving funding up to 10 lakhs.
  3. We also invite proposals for more than 10 Lakhs as major projects but these have to be preferably multicentric.
- The detailed proposals should include the following:
  - ◊ Title, names of principal and co-investigators, summary, introduction/background, review of literature, aims, methodology, study design, and detailed plan of work and bibliography. Brief biodata of principal investigator and other co-investigators

- ◊ Importance of work in the context of national priorities.  
Detailed budget sought along with full justification/ proposed utilization, of funding sought from RSSDI
- ◊ Whether the project is being partly funded from any other source?  
If yes, please mention the source and the amount received.
- ◊ Ethical committee clearance of the institution or other bonafide body.

## **Announcements**

Dear Member,

Please update your Membership details like Complete Postal Address, Email Id, Pan No. & Mobile no. after log in your membership area on our website [www.rssdi.in](http://www.rssdi.in) under sub heading Membership corner, so that we can send you RSSDI Newsletter & Journals.



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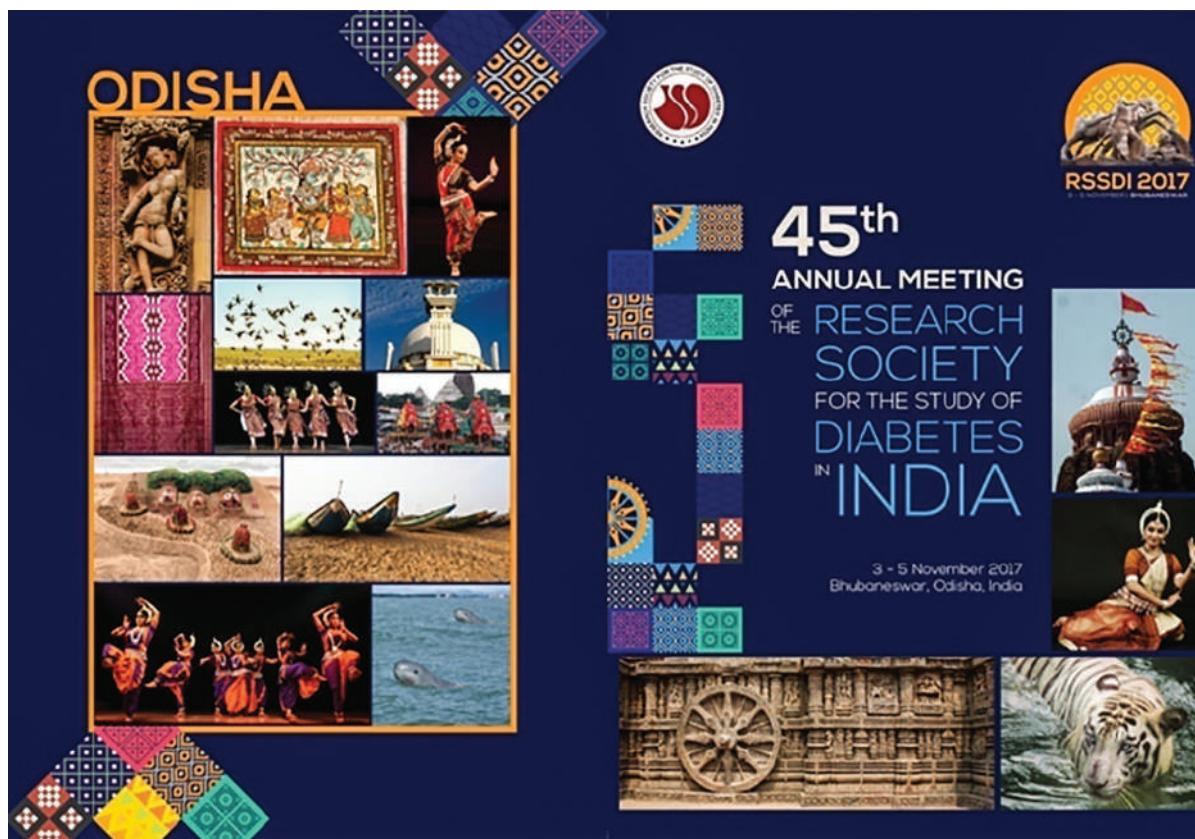
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## Invitation to the RSSDI,2017 Conference



Dear Friends,

We have great pleasure in inviting you to the 45th Annual Conference of Research Society for the Study of Diabetes in India (RSSDI) to be held in the smart city of Bhubaneswar from 3rd to 5th November 2017.

Bhubaneswar is the capital city of scenic Odisha. the soul of India which is naturally beautiful with forests. wildlife, sea beaches, heritage temples and many historical monuments. It is also known for its flavor of hospitality and varieties of delicious foods. We assure your convenient travel and comfortable stay with a relaxing time and a complete updated academic exposure.

The organizing committee will leave no stone unturned to make the event memorable for you and your family. please block your dates and register early to be a part of the event.

Wishing you a very happy. bright and prosperous new year ahead.

### **Team RSSDI 2017**

Bhubaneswar, Odisha



**Patron & Organizing Chairman**  
Prof. (Dr.) Sidhartha Das



**Organizing Secretary**  
Dr. Jayant Panda



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