



Contents available at ScienceDirect

Diabetes Research  
and Clinical Practicejournal homepage: [www.elsevier.com/locate/diabres](http://www.elsevier.com/locate/diabres)International  
Diabetes  
Federation

## Clinical features, biochemistry and HLA-DRB1 status in youth-onset type 1 diabetes in Pakistan



Asher Fawwad<sup>a,b,1</sup>, Denira Govender<sup>c,d,1</sup>, Mohammad Yakoob Ahmedani<sup>b</sup>, Abdul Basit<sup>b</sup>, Julie Ann Lane<sup>e</sup>, Steven John Mack<sup>e</sup>, Mark Alvin Atkinson<sup>f,g</sup>, Clive Henry Wasserfall<sup>f</sup>, Graham David Ogle<sup>c,h,\*</sup>, Janelle Annette Noble<sup>e</sup>

<sup>a</sup>Biochemistry Department, Baqai Medical University, Gadap, Karachi, Pakistan

<sup>b</sup>Baqai Institute of Diabetology and Endocrinology, Nazimabad, Karachi, Pakistan

<sup>c</sup>Life for a Child, Glebe, NSW 2037, Australia

<sup>d</sup>Sydney Medical School, University of Sydney, NSW 2006, Australia

<sup>e</sup>Children's Hospital Oakland Research Institute, Oakland, CA 94609, USA

<sup>f</sup>Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, Diabetes Institute, University of Florida, Gainesville, FL 32610, USA

<sup>g</sup>Department of Pediatrics, College of Medicine, Diabetes Institute, University of Florida, Gainesville, FL 32610, USA

<sup>h</sup>Diabetes NSW, Sydney, Australia

### ARTICLE INFO

#### Article history:

Received 22 October 2018

Received in revised form

10 December 2018

Accepted 15 January 2019

Available online 30 January 2019

#### Keywords:

Childhood diabetes

Pakistan

HLA

Autoantibody

C-peptide

### ABSTRACT

Published information on diabetes in Pakistani youth is limited. We aimed to investigate the demographic, clinical, and biochemical features, and HLA-DRB1 alleles in new cases of diabetes affecting children and adolescents <22 years of age.

The study was conducted at Baqai Institute of Diabetology and Endocrinology in Karachi from June 2013–December 2015. One hundred subjects aged <22 years at diagnosis were enrolled. Demographic characteristics, clinical information, biochemical parameters (blood glucose, HbA1c, C-peptide, glutamic acid decarboxylase 65 (GAD65) and islet antigen 2 (IA-2) autoantibodies) were measured. DNA from 100 subjects and 200 controls was extracted and genotyped for HLA-DRB1 using high-resolution genotyping technology.

Ninety-nine subjects were clinically diagnosed as type 1 diabetes (T1D) and one as type 2 diabetes (T2D). Of the 99 with T1D, 57 (57.6%) were males and 42 (42.4%) females, with mean age at diagnosis  $11.0 \pm 5.2$  years (range 1.6–21.7 years) and peaks at six and fifteen years. Fifty-seven subjects were assessed within one month of diagnosis and all within eleven months. For the subjects diagnosed as T1D, mean C-peptide was  $0.63 \pm 0.51$  nmol/L ( $1.91 \pm 1.53$  ng/mL), with 16 (16.2%) IA2 positive, 53 (53.5%) GAD-65 positive, and 10 (10.1%) positive for both autoantibodies. In T1D patients, the allele DRB1\*03:01

\* Corresponding author at: 22 Howard Pl, North Epping, NSW 2121, Australia.

E-mail addresses: [asherfawwad@bide.edu.pk](mailto:asherfawwad@bide.edu.pk) (A. Fawwad), [dgov8678@uni.sydney.edu.au](mailto:dgov8678@uni.sydney.edu.au) (D. Govender), [myakoob@bide.edu.pk](mailto:myakoob@bide.edu.pk) (M.Y. Ahmedani), [abdulbasit@bide.edu.pk](mailto:abdulbasit@bide.edu.pk) (A. Basit), [howlane@sbcglobal.net](mailto:howlane@sbcglobal.net) (J.A. Lane), [sjmack@chori.org](mailto:sjmack@chori.org) (S.J. Mack), [atkinson@ufl.edu](mailto:atkinson@ufl.edu) (M.A. Atkinson), [clive@ufl.edu](mailto:clive@ufl.edu) (C. Henry Wasserfall), [grahamo@diabetesnsw.com.au](mailto:grahamo@diabetesnsw.com.au) (G.D. Ogle), [jnoble@chori.org](mailto:jnoble@chori.org) (J.A. Noble).

<sup>1</sup> Indicates equal contribution to the paper and recognition as co-first author.

<https://doi.org/10.1016/j.diabres.2019.01.023>

0168-8227/© 2019 Elsevier B.V. All rights reserved.

demonstrated highly significant T1D association ( $p < 10^{-16}$ ), with no apparent risk conferred by DRB1\*04:xx alleles.

**Conclusions:** Heterogeneous forms of T1D appear more common in children and youth in Pakistan than in European populations. Individual understanding of such cases could enable improved management strategies and healthier outcomes.

© 2019 Elsevier B.V. All rights reserved.

## 1. Introduction

Youth onset diabetes has multiple etiologies with varied geographic distribution [1,2]. Considering this variation, it is important to understand the pathophysiology in each part of the world. From this, education and training of healthcare professionals can be tailored to target the local situation. Furthermore, improved understanding of disease heterogeneity will enable the stakeholders to ensure the optimum utilisation of available resources.

Little published information exists on diabetes in Pakistani young people, the sixth most populous nation in the world [3]. Two incidence studies were performed in Karachi some years ago, in 1989–93 [4] and 1990–1999 [2], suggesting low incidence rates compared to Caucasian populations [2]. Beyond this, Shera et al. [5] reported on clinical presentation and complications of a group of young people with type 1 diabetes (T1D) in Karachi, Pakistan's capital, in 2008. There was also a small study on diabetes autoimmunity markers [6] which discusses the difficulty of distinguishing diabetes types. Finally, Lone et al. [7] reported on rates of diabetic ketoacidosis.

We conducted a prospective study of 100 consecutive new cases of diabetes diagnosed in children and adolescents <22 years of age, investigating demographic, clinical, biochemical features, and HLA-DRB1 alleles. This study was conducted at the Baqai Institute of Diabetology and Endocrinology (BIDE) in Karachi, which was the first specialty-oriented diabetes centre to be established in Pakistan [8].

## 2. Methods

### 2.1. Study site

The study was conducted at The Baqai Institute of Diabetology and Endocrinology (BIDE) in Karachi, Pakistan, which is a private tertiary care unit. All procedures were approved by relevant Ethics Committees in Pakistan, the United States, and Australia and were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to enrolment in the study. HLA-DRB1 genotyping was performed at Children's Hospital Oakland Research Institute with IRB approval.

### 2.2. Study subjects

A total of 100 subjects <22 years of age at diabetes diagnosis were enrolled. All subjects were diagnosed in a consecutive

series from June 23, 2013 to December 15, 2015. Date of biochemical assessment for this study ranged from December 30, 2013 to December 23, 2015 such that 14% were assessed on the same day as diagnosis, 19% within one week, 24% one week to one month, 32% one to six months and 11% after six months (maximum eleven months) from diagnosis. One patient was classified as having type 2 diabetes (T2D) after enrolment. Data for this patient was excluded from the genetic analyses. A set of 200 control subjects were collected as controls for the HLA genotyping studies. Inclusion criteria for the controls included 1) no diabetes, 2) both parents born in Pakistan, 3) not related to a T1D patient, and 4) not related to another control subject. Age and gender were not included and this detail was not revealed to the genotyping laboratory.

### 2.3. Demographic data

Date of birth, sex, ethnicity, city and province of residence at diagnosis, date of diagnosis, distance from the clinic, as well as distance and travel time to the BIDE centre were recorded.

### 2.4. Clinical parameters

Diabetes was diagnosed according to standard World Health Organisation (WHO) criteria [9]. Determination of the type of diabetes was made by the local investigators according to available clinical features and history. The presence of polyuria, polydipsia, weight loss, malnutrition and ketoacidosis at the time of diagnosis were recorded. Ketoacidosis was defined as the presence of ketonuria as well as acidosis (anion gap calculation and/or measurement of arterial blood gases).

The following information pertaining to diabetes care was recorded for each subject: date commencement of insulin, use of oral hypoglycaemic agents, and other medications or treatment. History of other medical conditions, and family history of type 1 diabetes were also recorded. Body weight and height at diagnosis were measured by electronic scales and stadiometer respectively, with subjects wearing lightweight clothing and without shoes. Body Mass Index (BMI) was then calculated. BMI SD scores were calculated using the WHO standards for those <5 years of age [10], and for 5–19 years [11]. For those 19–21 years of age, BMI SD was calculated using an age of 19.0 years.

### 2.5. Sample collection

Peripheral blood was collected by venepuncture into vacutainer tubes on the day of assessment, after an overnight fast. Serum samples were spun down immediately and stored in a

–20 °C freezer. For genotyping, approximately 200ul of peripheral blood was preserved by mixing with DNAGard® blood (BioMatrica, San Diego, CA), then drying for storage and shipment. For controls, approximately 1 ml of saliva was mixed with DNAGard® saliva stabilizing reagent (BioMatrica, San Diego, CA) for storage and shipment.

## 2.6. Biochemical parameters and serology

For diabetes patients, blood glucose was measured at diagnosis with a Selectra Pro S ELITech Group (Dieren, Netherlands). HbA1c was collected on the day of study assessment, and assayed using a BioRad D-10 analyser (Biorad Laboratories Inc., Hercules, USA). Fasting C-peptide and autoantibodies against glutamic acid decarboxylase 65 (GAD65) and islet antigen 2 (IA-2) were measured from frozen samples by commercially available ELISA kits (IBL, Hamburg, Germany) in Pakistan. Fasting C-peptide was measured in ng/mL. GAD65 and IA-2 autoantibodies were considered positive if levels were  $\geq 30$  IU/mL based on the standard curve, according to manufacturer's recommendation. Similar ELISA formats have been challenged in Islet Autoantibody Standardization Programs with comparable sensitivity and specificity to radioimmunoassays [12].

## 2.7. HLA-DRB1 genotyping

Blood samples collected from patients and saliva samples from controls were preserved using DNAGard® reagents from BioMatrica, Inc. (San Diego, USA). DNA was extracted from preserved blood and saliva samples using QIAamp® blood kits (Qiagen). The samples were genotyped for HLA-DRB1 alleles with high-resolution genotyping technology at the Children's Hospital Oakland Research Institute in California. HLA sequence data for DRB1 exon 2 were generated using next-generation sequencing on the 454 GS Junior System (Roche, Basel, Switzerland) and on the MiSeq® platform (Illumina, San Diego, CA).

For the Roche 454 platform, PCR products (amplicons) were generated from genomic DNA using DRB generic, exon 2 454 fusion primers. The 454 fusion primers consist of a locus-specific primer on the 3' end, a 10-bp multiplex ID (MID) tag, and an "A" or "B" 454-specific sequence on the 5' end. Amplicons were purified with AMPure beads (Becton Dickinson, Franklin Lakes, USA), quantified using the Quant-iT PicoGreen dsDNA reagent (Life Technologies, Foster City, USA), and mixed with capture beads after dilution. Individual DRB exon 2 amplicon molecules were captured by these beads and amplified in an emulsion PCR. DNA-containing beads were pyrosequenced on the 454 GS Junior instrument to obtain sequence reads originating from a single molecule [13,14].

For the Illumina platform, DNA samples were amplified with primers containing the same locus-specific sequence used for the 454 platform but containing adapters to allow a second round of amplification. The second round of amplification served to attach individual "barcode" identifiers and sequences specific for sequencing on the Illumina platform. Second-round amplicons were purified and quantified as above, then mixed in equal proportions before sequencing on the MiSeq instrument.

DRB1 genotype calls for data generated on the Roche 454 platform were assigned with a combination of two customized software packages: Assign™ ATF (Conexio Genomics, Freemantle, Western Australia) and Sequence Compilation and REarrangement (SCORE™) software (Graz, Austria) [15]. Genotype calls for data generated on the Illumina MiSeq platform were assigned using HLA Twin™ software, version 2.1 (Omixon, Budapest, Hungary).

## 2.8. Statistics

The data were assessed for bimodality using likelihood ratio tests for nested finite mixture models. Models were fitted using an Expectation-Maximisation algorithm implemented in R 3.3.1 (R Core Team, Vienna, Austria) and with the package 'mixtools' [16]. Significance was assessed using Wilks' chi-square approximation and by parametric bootstrap with 1000 samples. To assess sensitivity to distributional assumptions, the analysis was performed in triplicate, assuming mixtures of Normal, LogNormal and Gamma populations in turn. Locus-level tests of heterogeneity and allele-level chi-squared ( $\chi^2$ ) tests of association between T1D subjects and control subjects were performed for the HLA-DRB1 locus using the BIGDAWG R package [17]. Alleles were analysed at the two-field (peptide) level. For each allele-level comparison, alleles with expected counts less than 5 in cases or controls were combined into a common "binned" category for analysis [18].

Hardy-Weinberg equilibrium (HWE) proportions of HLA-DRB1 genotypes in T1D subjects and control subjects were tested using PyPop (v0.8.0) [19]. The significance of locus-level HWE deviations was tested using Guo and Thompson's exact method [20], and individual genotypes deviating significantly from HWE expectations were identified using Chen's method [21,22], using a threshold of significance of 0.05. Graphs were created using Microsoft Excel software.

## 3. Results

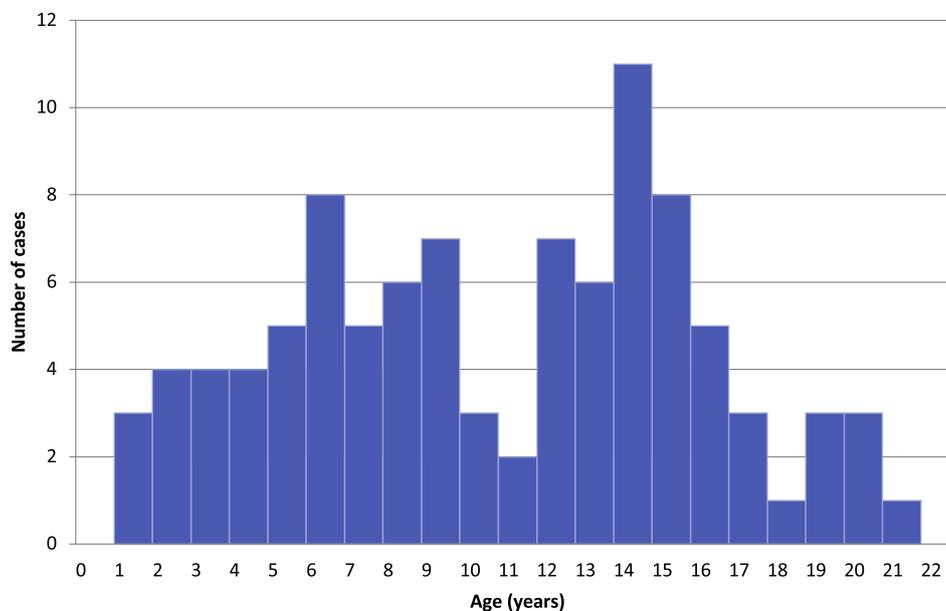
### 3.1. Diagnosis

Ninety-nine of the 100 enrolled diabetes patients were diagnosed as having T1D. One male who presented at 11.9 years of age was diagnosed with T2D.

### 3.2. Demographic characteristics

The 99 subjects with T1D included 57 (57.6%) males and 42 (42.4%) females. There was no discernible seasonal pattern in disease incidence (data not shown). 61.6% were of Muhajir ethnicity, 13.1% Sindhi, 10.1% Pashtun, 8.1% Punjabi, 2.0% Balochi and 5.1% other ethnic groups.

The mean  $\pm$  SD age of diagnosis of T1D was  $11.0 \pm 5.2$  years (range 1.6–21.7 years). The median age at diagnosis was 11.0 years (Fig. 1) with a suggestion of two peaks, the first at 6–10 years and the second at 12–16 years. On bimodality analysis, the binary mixture model was significantly preferred to the single population model ( $p < 0.05$ ) using both the Wilks' chi-square approximation and parametric bootstrap tests. We report the model with the most favourable Bayesian



**Fig. 1 – The age of onset of T1D in young people <22 years of age in Pakistan.**

Information Criterion. Under this model, 48% of the data come from a Normal distribution with mean 6.4 and SD 2.7, and 52% from a Normal distribution with mean 15.2 and SD 2.8.

Fifteen subjects (15.2%) were diagnosed at 0–4 years, 31 (31.3%) from 5 to 9 years, 29 (29.3%) from 10 to 14 years, 20 (20.2%) from 15 to 19 years and 4 (4.0%) 20–21 years. Of all 100 subjects, 41 (41%) travelled < 10 km to access care, 42 (42%) 10–50 km, 4 (4%) 50–200 km and 13 (13%) > 200 km.

### 3.3. Clinical parameters

The main symptoms preceding T1D diagnosis were polyuria ( $n = 98$ , 99.0%), polydipsia ( $n = 97$ , 98.0%) and weight loss ( $n = 99$ , 100%). Twenty-one (21.2%) presented in diabetic ketoacidosis (DKA).

For the 91 subjects with T1D who had both weight and height measured at diagnosis, the mean  $\pm$  SD BMI was  $16.5 \pm 3.3$  (range 10.2–27.3). Mean BMI standard deviation score (SDS) ranged from  $-7.41$  to  $+2.61$  (mean =  $-1.04$ ). Two subjects had a BMI SDS  $> 2:1$  2.61 in a boy aged 5 years with height SD  $-0.25$ , no DKA, both autoantibodies negative, and C-peptide 0.40 nmol/L (1.2 ng/mL) and 2) 2.27 in a boy aged 5 years with height SD  $-3.72$ , no DKA, both autoantibodies negative, and C-peptide 0.53 nmol/L (1.6 ng/mL). Two subjects had a BMI SDS  $< -5.0$ : 1)  $-6.18$  in a boy aged 14 years with height SD  $-2.33$ , with malnutrition, DKA present, autoantibodies negative, C-peptide 0.40 nmol/L (1.2 ng/mL); and 2)  $-7.41$  in a girl aged 16 years with height SD  $-1.80$ , malnutrition, no DKA, autoantibodies negative, C-peptide 0.93 nmol/L (2.8 ng/mL).

For T1D subjects, the mean  $\pm$  SD blood glucose at diagnosis was  $28.7 \pm 6.3$  mmol/L (range 13.6–45.9 mmol/L). The mean  $\pm$  SD HbA1c was  $12.7 \pm 2.7\%$  ( $115.0 \pm 29.4$  mmol/mol), with a range 7.1–21.0 (range 54.1–206.0 mmol/mol).

Four T1D subjects had other medical conditions: two with cerebral palsy, one with thalassaemia (diagnosed after the

diagnosis of diabetes), and one with both cerebral palsy and thalassaemia, diagnosed after diabetes. One T1D subject had a sister with T1D.

All T1D subjects were treated with insulin. Eighty-seven (87.9%) started on insulin on the same day as diagnosis, 10 (10.1%) within 1 week, and two (2.0%) within 2 weeks.

The single case diagnosed with T2D was a boy diagnosed at 12.0 years with a BMI SD of 2.78 and a height SD of 2.07, no DKA, both autoantibodies negative, and C-Peptide 1.62 nmol/L (4.9 ng/mL). He was initially commenced on insulin but then moved to metformin.

### 3.4. C-peptide

The mean  $\pm$  SD for C-peptide was  $0.63 \pm 0.51$  nmol/L ( $1.91 \pm 1.53$  ng/mL). Of T1D subjects, 3 (3.0%) had a C-peptide value  $< 0.13$  nmol/L ( $< 0.4$  ng/mL), 9 (9.1%) between 0.13 and 0.26 nmol/L ( $0.4$ – $< 0.8$  ng/mL), 64 (64.6%) between 0.26 and 0.66 nmol/L ( $0.8$ – $< 2.0$  ng/mL), 13 (13.1%) between 0.66 and 1.03 nmol/L ( $2.0$ – $< 3.1$  ng/mL), and 10 (10.1%)  $> 1.03$  nmol/L ( $> 3.1$  ng/mL). There was no relationship between C-peptide and duration of diabetes before assessment ( $r = 0.07$ ,  $p = 0.5$  on linear regression). Table 1 shows relationships with DKA and autoantibody status.

### 3.5. Autoantibody results

For T1D subjects, 16 (16.2%) were IA-2 autoantibody positive, 53 (53.5%) were GAD65 autoantibody positive, and 10 (10.1%) were positive for both autoantibodies (see Table 1 for further relationships). Amongst seropositive subjects, the mean  $\pm$  SD titre was  $105.8 \pm 137.0$  IU/mL for GAD65 autoantibodies and  $74.7 \pm 198.8$  IU/mL for IA-2 autoantibody levels. Table 1 shows relationships with DKA and C-peptide status.

**Table 1 – The relationships of diabetic ketoacidosis, C-peptide and autoantibodies for 99 T1D subjects.**

	C-peptide		Autoantibody status					
	C-peptide < 0.13 nmol/L (<0.4 ng/mL)	C-peptide 0.13–0.26 nmol/L (0.4–0.8 ng/mL)	C-peptide > 0.26–1.03 nmol/L (0.8–3.1 ng/mL) <sup>1</sup>	GAD ≥ 30	IA2 ≥ 30	Both autoantibodies	Either/both autoantibodies	Neither autoantibodies
	1 (5%)	1 (5%)	4 (19%)	11 (52%) 1 (33%)	3 (10%) 0 (0%)	2 (5%) 0 (0%)	12 (57%) 1 (33%)	9 (43%) 2 (67%)
Diabetic Ketoacidosis (n = 21)								
C-peptide < 0.13 nmol/L (<0.4 ng/mL) (n = 3)	1 (5%)							
C-peptide 0.13–0.26 nmol/L (0.4–0.8 ng/mL) (n = 9)		15 (71%)		4 (44%)	1 (11%)	0 (0%)	5 (56%)	4 (44%)
C-peptide 0.26–1.03 nmol/L (0.8–3.1 ng/mL) <sup>2</sup> (n = 77)			4 (19%)	42 (55%)	13 (17%)	8 (10%)	47 (61%)	30 (39%)
C-peptide > 1.03 nmol/L (>3.1 ng/mL) (n = 10)				6 (60%)	2 (20%)	2 (20%)	6 (60%)	4 (40%)

<sup>1</sup> For those who presented within one month in 16 subjects, 1–4 months in 4 subjects, and eight months in one subject.

<sup>2</sup> Normal range.

### 3.6. HLA-DRB1 results

HLA-DRB1 genotypes were generated successfully for 100 patients and 188 control subjects. Association analysis for 99 T1D patients and 188 control subjects is shown in Table 2. As expected, the association of the DRB1 locus with T1D was highly significant ( $p < 2.22 \times 10^{-16}$ ). The risk for T1D was primarily driven by the allele DRB1\*03:01, which is part of the conserved DRB1\*03:01-DQA1\*05:01-DQB1\*02:01 haplotype (sometimes abbreviated as DR3-DQ2). DRB1\*03:01 was seen at 51% frequency in T1D subjects but only 12% in controls (OR = 7.86; 95% CI = 5.06–12.25;  $p < 2.22 \times 10^{-16}$ ). The less frequent DRB1\*14:04 allele (observed in 6.6% of T1D subjects but only 1.6% of controls) also predisposed to T1D (OR = 4.33; 95% CI = 1.5–14.1;  $p = 1.56E-03$ ). Coincidentally, both alleles are roughly four times more frequent in T1D patients than controls (DRB1\*03:01: 4.4x; DRB1\*14:04: 4.1x). In Asians, DRB1\*14:04 is commonly found in a haplotype with DQB1\*05:01. This haplotype was not found to be significantly associated with T1D in the Type 1 Diabetes Consortium data [23], but is significantly positively associated with juvenile autoimmune thyroiditis in North India [24]. Notably, the similar European haplotype DRB1\*14:01-DQB1\*05:01 exhibits a very strong protective association with T1D [23]. As anticipated, the Asian allele DRB1\*04:03 was significantly negatively associated with T1D. Other DRB1\*04 alleles, particularly DRB1\*04:01/02/04/05, which are usually positively associated with T1D, were rare in these data and not significantly predisposing for T1D. Of the T1D patients, 66.7% contained at least one copy of DRB1\*03:01, and 35.4% were homozygous for the DRB1\*03:01 allele. This is in stark contrast to the typical HLA association result seen in European-derived populations, where up to 40% of patients are heterozygous for DRB1\*03:01 and DRB1\*04:01/04:02/04:04/04:05/04:08 [23].

While DRB1 genotype proportions of the control subjects comported to HWE expectations ( $p = 0.35$ ), DRB1 genotypes in the T1D subjects displayed an overall HWE deviation ( $p = 4.6 \times 10^{-05}$ ) characterized by an excess of homozygotes (39 observed, 27.57 expected,  $p = 0.03$ ). In particular, a significant excess of DRB1\*03:01 + DRB1\*03:01 homozygous genotypes (35 observed, 25.8 expected,  $p = 2.5 \times 10^{-04}$ ) and significant decrease in DRB1\*03:01 heterozygotes (31 observed, 49.5 expected,  $p = 8.6 \times 10^{-03}$ ) was observed for T1D subjects. Deviations were not observed for these genotypes in control subjects (DRB1\*03:01 homozygotes: 4 observed, 2.57 expected,  $p = 0.47$ ; DRB1\*03:01 heterozygotes: 36 observed, 38.85 expected,  $p = 0.65$ ).

## 4. Discussion

This study investigated the clinical, biochemical, and genetic characteristics of new and recent onset diabetes in children and youth in Pakistan.

The pattern of age of onset of subjects diagnosed with T1D suggested a bimodal distribution with peak diagnosis at approximately six to nine years in age with a second peak occurring in the early teenage years, a finding similar to the pattern observed in some other studies [25,26]. However, previous studies in Pakistan have reported the peak age of T1D

onset as 10–14 years [2] and 10–12 years [4]. The male excess seen in this study was also observed in Karachi by Shera et al. [5], and in some studies in northern India [27–29]. However, Staines et al. [4] in Karachi found a slight female excess as did and some studies in northern India. Globally, low incidence countries tend to have a female excess [30].

Understanding of T1D in Pakistan is hampered by the lack of recent incidence studies. The two past studies suggested low incidence rates: 1.0 cases per 100,000 children < 17 years of age per year in Karachi between 1989 and 1993 [4], and 0.5 cases per 100,000 children < 15 years of age per year in Karachi from 1990 to 1999 [2] compared to rates of 4.2–40.9 in the European populations reported in the same period [2].

In our study, most T1D subjects (61.6%) were from the Muhajir ethnic group, who came from northern India at the time of the partition with Pakistan in 1947. This group represents only 7.6% of Pakistan's population [3] but in Karachi, it is estimated to be 50% of the population [31].

The incidence of T1D can change in migrant populations. The reported incidences in Karachi (with 50% of Indian descent) are lower than those reported in India: 3.0 per 100,000 < 15 years in Karnal (northern India) in 2008 [27,32] and 10.5 in 1991–1994 in Madras (southern India) [33]. In children of South Asian migrants to the United Kingdom and Sweden, rates are substantially higher than those observed in Pakistan or northern India but less than those in children born to parents from the country of immigration. For example, in Yorkshire (United Kingdom), the rate was 14.7 per 100,000 children < 15 years of age of South Asian parents compared to 21.5 for non-south Asians [34] and in Sweden, the rate in children of South Asians was 34% of that in children of parents born in Finland (with an overall rate of 29 per 100,000 < 15 years of age) [35]. Such data show the potent

impacts of both genetic and environmental effects in incidence of T1D [1,36].

In this cohort, all but one of the 100 patients were clinically diagnosed with T1D. However, the data suggest that those classified as T1D are a heterogeneous group, consisting of typical/classic T1D, atypical T1D, and possibly other forms of diabetes such as malnutrition-related diabetes and T2D. The rate of DKA at onset was 21.2%, which is relatively low by international standards [37]. The two previous Pakistan studies reported markedly different rates: 57.2% [7] and 5.8% [5]. The GAD65 autoantibody positivity of 54% and IA-2 autoantibody positivity of 16% (with 10.1% of all 99 cases having both autoantibodies) are also relatively low [38]. Only 12.1% had a fasting C-peptide at diagnosis of 0.25 nmol/L (< 0.8 ng/mL), which is typically the upper threshold for insulin requiring T1D [39] and in 10.1% of cases, the C-peptide was > 1.03 nmol/L (> 3.1 ng/mL).

Such heterogeneity of those diagnosed with T1D could be partly due to incomplete characterisation. Further autoantibody studies, of insulin autoantibodies and particularly Zinc transporter 8 (ZnT8), could reveal unrecognised autoimmunity. Shivaprasad et al. [40] documented rates of 64.7%, 31.8% and 19.3% for GAD-65, ZnT8 and IA-2 autoantibodies, respectively, with one quarter of those positive for ZnT8 being negative for the other two autoantibodies. However, autoantibody rates lower than those seen in children of European descent are not uncommon in studies from India [41–45]. Even in Western countries, some T1D patients are autoantibody negative; in the SEARCH study for instance, 15.6% of those who had diabetes and were insulin sensitive were autoantibody negative [46]. C-peptide studies after the honeymoon period has passed could show whether some of these subjects just have a slowly-progressive T1D, T2D, or possibly a different type of diabetes that is neither T1D nor T2D. T2D occurs at

**Table 2 – Association of DRB1 Alleles with Type 1 Diabetes in Pakistani Patients and Controls.**

Allele	Controls		Patients		OR	95% C.I.	p-value	Significance <sup>1</sup>
	Count	Frequency	Count	Frequency				
DRB1*01:01	15	0.03989	8	0.04040	1.01	0.37–2.6	9.76E-01	NS
DRB1*03:01	44	0.11702	101	0.51010	7.86	5.06–12.25	< 2.22E-16	*
DRB1*04:03	16	0.04255	1	0.00505	0.11	0–0.75	1.18E-02	*
DRB1*07:01	61	0.16223	12	0.06061	0.33	0.16–0.65	5.13E-04	*
DRB1*09:01	9	0.02394	9	0.04545	1.94	0.67–5.62	1.60E-01	NS
DRB1*10:01	21	0.05585	5	0.02525	0.44	0.13–1.22	9.38E-02	NS
DRB1*11:01	27	0.07181	5	0.02525	0.33	0.1–0.9	2.08E-02	*
DRB1*11:04	17	0.04521	2	0.01010	0.22	0.02–0.92	2.54E-02	*
DRB1*13:01	31	0.08245	5	0.02525	0.29	0.09–0.77	7.22E-03	*
DRB1*14:04	6	0.01596	13	0.06566	4.33	1.5–14.1	1.56E-03	*
DRB1*15:01	26	0.06915	3	0.01515	0.21	0.04–0.69	4.99E-03	*
DRB1*15:02	32	0.08511	2	0.01010	0.11	0.01–0.44	2.96E-04	*
Binned	71	0.18883	32	0.16163	0.83	0.51–1.33	4.19E-01	NS
Total	376		198					

OR: Odds Ratio.

CI: Confidence Interval.

Binned: Alleles with expected counts less than five in cases or controls were combined into a common “Binned” category for analysis, as described in Methods section 1.9. Counts and frequencies for all detected alleles are included in Supplementary Table S1.

1: The p-values for DRB1 alleles were not corrected for the number of comparisons, as the DRB1 locus displayed significant overall heterogeneity (p-value < 2.22E-16). Significant p-values are indicated with asterisks. NS. Not significant

Total: The total number of alleles identified in controls and patients is shown in the last row.

lower BMIs in Asian as compared to European populations [47]. According to the Second National Diabetes Survey of Pakistan in 2016–17, rates of T2D in adults  $\geq 20$  years of age in Pakistan are 26.3% (males 26.2% and females 26.3%) [48]. The incompletely understood and possibly overlapping entities of malnutrition-related diabetes and fibrocalculous pancreatic disease (FCPD) [49] are seen in India and Bangladesh [41,42,49], but have not been reported from Pakistan. None of the subjects in this study had the history of chronic abdominal pain that is characteristic of FCPD, although two subjects had a BMI SDS  $< 5.0$  and so it is possible that malnutrition was occurring. Complicating diagnosis further, these two conditions may also have autoantibodies [42].

In a small study from northern Pakistan, Fatima et al. [6] found that distinguishing between T1D and T2D was difficult even when autoantibodies and insulin resistance were studied. For individual subjects, it is important to be able to recognise if diabetes is not a classic T1D pattern with very low insulin secretion. Inexpensive oral medicines such as metformin or gliclazide may be of added benefit in a subset of individuals. If good control can be achieved from such medicines, then insulin may not be needed.

The T1D patients in this study seem to have an unusually high representation of DRB1\*03:01, with more homozygotes than expected. Kelly et al. [50] studied the T1D immunological features in Indo-Aryan children, with both parents originally from Pakistan or Indian Punjab, residing in the UK and found that the DR3-DQ2 haplotype (DRB1\*03:01-DQA1\*05:01-DQB1\*02:01) was significantly positively associated with T1D in this population, as it is in most populations [24]. Rasmussen et al. [51] examined HLA in newborns of Pakistani migrants in Norway to look for DR-DQ haplotypes considered to confer high-risk for T1D. They found the DR3-DQ2 haplotype in high frequency (18.5%); no instances of DR3/4 heterozygotes with a predisposing DR4 (expectation would be 2 or 3), and an excess of DR3/DR3 homozygous individuals ( $n = 13$ , with expectation of 6). These data are somewhat consistent with the results presented here; however, because the study was of newborns, diabetes status was unable to be determined.

Data from a population of Somali immigrants to the United States were quite similar to our Pakistani data in that nearly all of the T1D susceptibility was attributable to DRB1\*03:01. Nearly every patient genotype (93%) included at least one DRB1\*03:01 allele, and 50% of patients were homozygotes for DRB1\*03:01 [52]. One interpretation of this observation could be that the Pakistani and Somalian populations may partly share a common ancestry and genetic predisposition for T1D. Further genotyping of the HLA region as well as other T1D susceptibility loci in both populations will address this issue.

Funding constraints limited this study to two autoantibodies (GAD65 and IA-2), a single HLA gene (DRB1), and a one-time measurement of C-peptide, with no external quality control. When funds are identified, samples will be genotyped for all classical HLA loci. Expansion to include other autoantibodies (particularly ZnT8) [40] and other HLA and genetic loci [53] could reveal further information. Longitudinal studies of C-peptide and insulin requirements would help further discrimination of the types of diabetes occurring in this population. Another limitation is that C-peptide and autoantibodies

were not always measured at diagnosis. Particularly for C-peptide, this may have resulted in a few subjects being in the “honeymoon phase” with some recovery of insulin secretion.

In conclusion, this study shows that children and adolescents diagnosed with type 1 T1D in Pakistan may be a heterogeneous group, as GAD-65 and IA2 autoantibody positivity and low C-peptide values are less common than in populations of European ancestry. HLA-DRB1 shows highly significant T1D association, but the overall allele distribution and individual allele associations differ from Europeans, with DRB1\*03:01 showing exceptionally strong risk. If there is greater heterogeneity, individualised assessment of atypical “T1D” cases could lead to improved management strategies and healthier outcomes.

## Acknowledgements

The authors thank Angela Middlehurst for helping with the ethics application, Amanda Posgai for her aid in composing the manuscript, Sarah Garnett for the BMI SD data, and Gabriel Gregory for helping with statistical analysis. We appreciate the support of IBL, who provided the ELISA kits at a reduced cost. This study was partly funded by the Leona M. and Harry B. Helmsley Charitable Trust, and was supported by National Institutes of Health (NIH) National Institute of Allergy and Infectious Disease (NIAID) grant R01AI28775 (SM). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the Leona M. and Harry B. Helmsley Charitable Trust, NIAID, NIH or United States Government.

No potential conflicts of interest relevant to this article were reported.

AF, MYA and AB implemented the study in Pakistan and helped with writing the manuscript. DG did the data analysis and wrote the initial draft of the manuscript. MAA and CHW advised on the study protocol, implementation, and analysis and contributed to the manuscript. SM performed statistical analysis of HLA data, and JL performed HLA genotyping. GDO designed and coordinated the study and co-wrote the manuscript. JN designed and coordinated the genetic portion of the study, including sample collection, DRB1 genotyping, and HLA analysis, and co-wrote the manuscript.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.diabres.2019.01.023>.

## REFERENCES

- [1] Craig ME, Jefferies C, Dabelea D, Balde N, Seth A, Donaghue KC. Definition, epidemiology, and classification of diabetes in children and adolescents. *Pediatric Diabetes* 2014;15(Suppl. 20):4–17.
- [2] DIAMOND Project Group. Incidence and trends of childhood type 1 diabetes worldwide 1990–1999. *Diabet Med* 2006;23:857–66.

- [3] Central Intelligence Agency World Factbook. The World Factbook, South Asia: Pakistan, 2018. Retrieved May 31, 2018 from <https://www.cia.gov/library/publications/resources/the-world-factbook/geos/pk.html>.
- [4] Staines A, Hanif S, Ahmed S, McKinney PA, Shera S, Bodansky HJ. Incidence of insulin dependent diabetes mellitus in Karachi, Pakistan. *Arch Dis Child*. 1997;76(2):121–3.
- [5] Shera AS, Miyan Z, Basit A, Maqsood A, Ahmadani MY, Fawwad A, et al. Trends of type 1 diabetes in Karachi. Pakistan. *Pediatr Diabetes*. 2008;9(4 Pt 2):401–6. <https://doi.org/10.1111/j.1399-5448.2007.00309.x>.
- [6] Fatima A, Khawaja KI, Burney S, Minhas K, Mumtaz U, Masud F. Type 1 and type 2 diabetes mellitus: are they mutually exclusive? *Singapore Med J* 2013;54(7):396–400.
- [7] Lone SW, Siddiqui EU, Muhammed F, Atta I, Ibrahim MN, Raza J. Frequency, clinical characteristics and outcome of diabetic ketoacidosis in children with type-1 diabetes at a tertiary care hospital. *J Pak Med Assoc* 2010;60:725–9.
- [8] Basit A, Riaz M, Fawwad A. Improving diabetes care in developing countries: the example of Pakistan. *Diabetes Res Clin Pract* 2015;107(2):224–32. <https://doi.org/10.1016/j.diabres.2014.10.013>. Epub 2014 Oct 24.
- [9] World Health Organization, & International Diabetes Federation, 2006. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia. Retrieved February 11, 2018, from [http://www.who.int/diabetes/publications/Definition%20and%20diagnosis%20of%20diabetes\\_new.pdf](http://www.who.int/diabetes/publications/Definition%20and%20diagnosis%20of%20diabetes_new.pdf).
- [10] WHO Multicentre Reference Study Group. WHO child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development. *Acta Paediatr Suppl* 2006;450:76–85.
- [11] de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. *Bull World Health Organ* 2007;85(9):660–7.
- [12] Wasserfall C, Montgomery E, Yu L, Michels A, Gianani R, Pugliese A, et al. Validation of a rapid type 1 diabetes autoantibody screening assay for community-based screening of organ donors to identify subjects at increased risk for the disease. *Clin Exp Immunol* 2016;185(1):33–41. <https://doi.org/10.1111/cei.12797>. Published online 2016 May 4.
- [13] Bentley G, Higuchi R, Högglund B, et al. High-resolution, high-throughput HLA genotyping by next-generation sequencing. *Tissue Antigens* 2009;74:393–403.
- [14] Holcomb CL, Högglund B, Anderson MW, et al. A multi-site study using high-resolution HLA genotyping by next generation sequencing. *Tissue Antigens* 2011;77:206–17.
- [15] Helmberg W, Lanzer G, Zahn R, Weinmayr B, Wagner T, Albert E. Virtual DNA analysis – a new tool for combination and standardised evaluation of SSO, SSP and sequencing-based typing results. *Tissue Antigens* 1998;51(6):587–92.
- [16] Benaglia T, Chauveau D, Hunter DR, Young D. mixtools: An R package for analysing finite mixture models. *J Stat Softw* 2009;32:1–29.
- [17] Pappas DJ, Marin W, Hollenbach JA, Mack SJ. Bridging ImmunoGenomic Data Analysis Workflow Gaps (BIGDAWG): An integrated case-control analysis pipeline. *Hum Immunol*. 2016;77(3):283–7. <https://doi.org/10.1016/j.humimm.2015.12.006>. Epub 2015 Dec 18.
- [18] Hollenbach JA, Mack SJ, Thomson G, Gourraud PA. Analytical methods for disease association studies with immunogenetic data. *Methods Mol Biol* 2012;882:245–66. [https://doi.org/10.1007/978-1-61779-842-9\\_14](https://doi.org/10.1007/978-1-61779-842-9_14).
- [19] Lancaster AK, Single RM, Solberg OD, Nelson MP, Thomson G. PyPop update—a software pipeline for large-scale multilocus population genomics. *Tissue Antigens* 2007;69(Suppl 1):192–7.
- [20] Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*. 1992;48(2):361–72.
- [21] Chen JJ, Thomson G. The variance for the disequilibrium coefficient in the individual Hardy-Weinberg test. *Biometrics*. 1999;55(4):1269–72.
- [22] Chen JJ, Hollenbach JA, Trachtenberg EA, et al. Hardy-Weinberg testing for HLA class II (DRB1, DQA1, DQB1, and DPB1) loci in 26 human ethnic groups. *Tissue Antigens* 1999;54(6):533–42.
- [23] Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P, et al. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* 2008;57(4):1084–92. <https://doi.org/10.2337/db07-1331>. Epub 2008 Feb 5.
- [24] Kanga U, Tandon N, Marwaha RK, Khanna R, Bhattacharya B, Singh S, et al. Immunogenetic association and thyroid autoantibodies in juvenile autoimmune thyroiditis in North India. *Clin Endocrinol (Oxf)* 2006;64:573–9.
- [25] Sterky G, Holmgren H, Gustavson KH, Larsson Y, Lundmark KM, Nilsson KO, et al. The incidence of diabetes mellitus in Swedish children 1970–1975. *Acta Paediatr Scand* 1978;67:139–43.
- [26] Demir F, Günöz H, Saka N, Darendeliler F, Bundak R, Baş F, et al. Epidemiological features of type 1 diabetic patients between 0 and 18 years of age in Istanbul City. *J Res Clin Pediatr Endocrinol* 2015;7:49–56.
- [27] Kalra S, Kalra B, Sharma A. Prevalence of type 1 diabetes mellitus in Karnal district, Haryana state, India. *Diabetol Metab Syndr* 2010;9(2):14. <https://doi.org/10.1186/1758-5996-2-14>.
- [28] Dayal D, Saini AG, Jayashree M, Singhi S, Kumar R, Samprati M, et al. Hospital based incidence, patterns of presentation and outcome of type 1 diabetes: 12 years' data from a tertiary care center in North India. *Int J Diabetes Dev Ctries* 2015;35:103–7.
- [29] Bhatia V, Arya V, Dabadghao P, Balasubramanian K, Sharma K, Verghese N, et al. Etiology and outcome of childhood and adolescent diabetes mellitus in North India. *J Pediatr Endocrinol Metab* 2004;17(7):993–9.
- [30] Karvonen M, Pitkaniemi M, Pitkaniemi J, Kohtamäki K, Simola E, Tajima N, et al. Sex-difference in the incidence of insulin-dependent diabetes mellitus. An analysis of the recent epidemiological data. *Diab Metab Rev* 1997;13:275–91.
- [31] World Population Review. (2018-05-20). Pakistan population; 2018. Retrieved May 20, 2018 from <http://worldpopulationreview.com/countries/pakistan-population/>.
- [32] International Diabetes Federation. IDF diabetes atlas, 8th ed. Brussels: International Diabetes Federation; 2017.
- [33] Ramachandran A, Snehalatha C, Krishnaswamy CV. Incidence of IDDM in children in urban population in southern India. Madras IDDM Registry Group Madras, South India. *Diabetes Res Clin Pract* 1996;34(2):79–82.
- [34] Harron KL, McKinney PA, Feltbower RG, Bodansky HJ, Norman PD, Campbell FM, et al. Incidence rate trends in childhood type 1 diabetes in Yorkshire, UK 1978–2007: effects of deprivation and age at diagnosis in the South Asian and non-South Asian populations. *Diabet Med*. 2011;28(12):1508–13. <https://doi.org/10.1111/j.1464-5491.2011.03413.x>.
- [35] Ji J, Hemminki K, Sundquist J, Sundquist K. Ethnic differences in incidence of type 1 diabetes among second generation immigrants and adoptees from abroad. *J Clin Endocrinol Metab*. 2010;95(2):847–50. <https://doi.org/10.1210/jc.2009-1818>. Epub 2009 Dec 18.

- [36] Katsarou A, Gudbjörnsdóttir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. *Nat Rev Dis Primers* 2017;30(3):17016. <https://doi.org/10.1038/nrdp.2017.16>.
- [37] Usher-Smith JA, Thompson M, Ercole A, Walter FM. Variation between countries in the frequency of diabetic ketoacidosis at first presentation of type 1 diabetes in children: a systematic review. *Diabetologia*. 2012;55(11):2878–94. <https://doi.org/10.1007/s00125-012-2690-2>. Epub 2012 Aug 30.
- [38] Writing Group for the SEARCH for Diabetes in Youth Study Group, Dabelea D, Bell RA, D'Agostino Jr RB, Imperatore G, Johansen JM, et al. Incidence of diabetes in youth in the United States 2007;vol. 297, no. 24:2716–24.
- [39] Jones AG, Hattersley AT. The clinical utility of C-peptide measurement in the care of patients with diabetes. *Diabet Med* 2013;30(7):803–17. <https://doi.org/10.1111/dme.12159>.
- [40] Shivaprasad C, Mittal R, Dharmalingam M, Kumar PK. Zinc transporter-8 autoantibodies can replace IA-2 autoantibodies as serological marker for juvenile onset type 1 diabetes in India. *Indian J Endocrinol Metab* 2014;18(3):345–9. <https://doi.org/10.4103/2230-8210.131174>.
- [41] Goswami R, Kochupillai N, Gupta N, Kukreja A, Lan M, Maclaren NK. Islet cell autoimmunity in youth onset diabetes mellitus in northern India. *Diabetes Res Clin Pract*. 2001;53(1):47–54.
- [42] Singh AK, Bhatia E, Dabadghao P, Bhatia V, Gellert A, Colman PG. Role of islet autoantibody in the aetiology of different clinical subtypes of diabetes mellitus in young north Indians. *Diabet Med*. 2000;17(4):275–80.
- [43] Balasubramanian K, Dabadghao P, Bhatia V, Colman PG, Gellert SA, Bharadwaj U, et al. High frequency of type 1B (idiopathic) diabetes in North Indian children with recent-onset diabetes. *Diabetes Care*. 2003;26(9):2697.
- [44] Dayal D, Samprati M, Kaur N, Minz RW, Jayaraman D. Prevalence of beta-cell, thyroid and celiac autoimmunity in North Indian children with recent onset type 1 diabetes (T1D). *J Clin Diagn Res* 2015;9(3):SM01-2. <https://doi.org/10.7860/JCDR/2015/11960.5711>. Epub 2015 Mar 1.
- [45] Tandon N, Shtauvere-Brameus A, Hagopian WA, Sanjeevi CB. Prevalence of ICA-12 and other autoantibodies in north Indian patients with early-onset diabetes. *Ann N Y Acad Sci*. 2002;958:214–7.
- [46] Dabelea D, Pihoker C, Talton JW, D'Agostino Jr RB, Fujimoto W, Klingensmith GJ, et al. Etiological approach to characterization of diabetes type: the SEARCH for Diabetes in Youth Study. *Diabetes Care* 2011;34(7):1628–33. <https://doi.org/10.2337/dc10-2324>. Epub 2011 Jun 2.
- [47] Chiu M, Austin PC, Manuel DG, Shah BR, Tu JV. Deriving ethnic-specific BMI cutoff points for assessing diabetes risk. *Diabetes Care* 2011;34(8):1741–8. <https://doi.org/10.2337/dc10-2300>. Epub 2011 Jun 16.
- [48] Basit A, Fawwad A, Qureshi H, Shera AS. NDSP Members. Prevalence of diabetes, pre-diabetes and associated risk factors: Second National Diabetes Survey of Pakistan (NDSP), 2016–2017. *BMJ Open* 2018;8(8):e020961. <https://doi.org/10.1136/bmjopen-2017-020961>.
- [49] Unnikrishnan R, Mohan V. Fibrocalculous pancreatic diabetes (FCPD). *Acta Diabetol*. 2015;52(1):1–9. <https://doi.org/10.1007/s00592-014-0685-9>. Epub 2014 Nov 14.
- [50] Kelly MA, Alvi NS, Croft NJ, Mijovic CH, Bottazzo GF, Barnett AH. Genetic and immunological characteristics of type 1 diabetes mellitus in an Indo-Aryan population. *Diabetologia* 2000;43(4):450–6.
- [51] Rasmussen T, Yap SE, Stray-Pedersen B, Akselsen HE, McKinney PA, Rønningen KS. HLA associated type 1 diabetes risk in children of Pakistani migrants to Norway. *Med Hypotheses* 2014;83(6):664–7. <https://doi.org/10.1016/j.mehy.2014.09.013>. Epub 2014 Oct 17.
- [52] Sunni M, Noble JA, Yu L, Mahamed Z, Lane JA, Dhunkal AM, et al. Predominance of DR3 in Somali children with type 1 diabetes in the twin cities. *Minnesota Pediatr Diabetes* 2017;18(2):136–42. <https://doi.org/10.1111/pedi.12369>. Epub 2016 Feb 8.
- [53] Kiani AK, John P, Bhatti A, Zia A, Shahid G, Akhtar P, et al. Association of 32 type 1 diabetes risk loci in Pakistani patients. *Diabetes Res Clin Pract*. 2015;108(1):137–42. <https://doi.org/10.1016/j.diabres.2015.01.022>. Epub 2015 Jan 21.