ORIGINAL ARTICLE

Validation of a screening tool for identifying Brazilians with impaired glucose tolerance

Bruno Pereira de Moura & Paulo Roberto do Santos Amorim & Sylvia do Carmo Castro Franceschini & Janice Sepúlveda Reis & João Carlos Bouzas Marins

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Abstract This study was designed to validate a self-assessment tool for identifying Brazilians with Impaired Glucose Tolerance (IGT). The Finnish Diabetes Risk Score (FINDRISC) was applied in 829 people aged over 40 years without previous diagnosis of diabetes between November 2009 and April 2010 at the Viçosa city, Minas Gerais, Brazil. We randomly selected 300 subjects in the initial survey, which received by post a letter of invitation to attend the laboratory for the collection of the blood sample to identify levels of glycated hemoglobin (A1C). Of these 300 subjects invited, 162 attended subjects, this being the final sample for the validation study. The risk score was evaluated through the Area Under the Curve (AUC) in a Receiver Operating Characteristics (ROC) curve. We assume a maximum error of 5 % and confidence interval 95 %. The prevalence of IGT and diabetes according to the A1C test were 21.6 % and 8.6 % respectively. The Diabetes Risk Score value varied from 1 to 25. The AUC by considering all values of A1C ≥ 6.0 % was 0.69 (95 % CI 0.61–0.76). The score value ≥ 9 had sensitivity of 75.51 %, specificity of 49.56 %, Positive Predictive Value (PPV) of 39.4 % and Negative Predictive Value (NPV) of 82.4 %. The FINDRISC is a suitable tool to identify Brazilians with IGT.

Keywords Type 2 diabetes · Screening · Impaired glucose tolerance · Assessment tool

P. R. S. Amorim (*)
e-mail: pramorim@ufv.br

Introduction

A recent study by the Ministry of Health [1] shows that the prevalence of diabetes in Brazil increased from 5.2 % in 2006 to 5.8 % in 2009. According to International Diabetes Federation (IDF) in 2010 [2], the estimated prevalence was 6.4 %, meaning 7,632,500 people with diabetes across the country and placing Brazil as the fifth country with the largest number of people with diabetes [3]. In 2030, it is estimated that the prevalence will rise to 7.7 % of the population [4].

The growing number of people with diabetes in their productive age increases the economic burden on the health care system, due to increasingly early onset of complications and subsequently a period of intensive long medical treatment [5].

Based on this evidence, identifying individuals with high risk for developing diabetes (IGT) or those already with the disease is extremely important [6]. However, the American Diabetes Association (ADA) [7] recommends that the tests to detect type 2 diabetes and assess risk for future diabetes in asymptomatic people should only be considered in adults with some additional risk factors.

Thus, in order to identify individuals at high risk of developing type 2 diabetes several simple, inexpensive and non-invasive tools were developed and tested in many populations over the last decade [8–11]. Such tools cannot be applied to other ethnic groups, except when validated for the targeted population [12]. Such predicting tools are lacking in the Brazilians populations. Therefore, the current study was conducted to validate a self-assessment tool for identifying Brazilians with IGT.

Participants and methods

Methods

This cross-sectional survey was conducted between November 2009 and April 2010 at the Viçosa city, Minas Gerais. The questionnaire to screen for diabetes was applied in 829 individuals (≥ 40 years) without previous diagnosis of diabetes. Questionnaires were administered by health workers in 15 units of the Family Health Program of the city and seven points at the university campus. Health workers involved in collecting data were trained and the equipment used for measuring body weight and height of the subjects had been tested and calibrated. Waist circumference was measured at the navel, according to the instructions of the questionnaire [13].

Subjects

The Viçosa city has about 72,220 inhabitants (51.5 % women). Approximately 26,133 (36.2 %) people are more than 40 years of age [14]. Currently, according to International Diabetes Federation (IDF) [2], the estimated prevalence of diabetes in Brazil is 6.4 %. Based on this information, we used the equation proposed by Lwanga and Lemeshow [15] for calculating the minimum sample in order to extract a sub-sample for the study of the questionnaire. Assuming a maximum error of 5 % and confidence interval 95 %, at least 117 individuals were required to the sub-sample representative of the population in question. However, in this analysis, the minimum was extrapolated to 162 individuals (an increase of approximately 40 %) to prevent sample loss during the study.

In summary, of the 829 subjects in the cross-sectional survey to screen for diabetes, 14 were excluded due to missing data, leaving 815 subjects (36.3 % men and 63.3 % women, 55.5±8.1 years and 26.9±3.7 BMI) for analysis. Among subjects with complete data, 300 subjects were randomly selected and received by post a letter of invitation to attend the Human Performance Laboratory at the University for blood collection in order to compose the sub-sample validation of a screening tool. Of these 300 subjects invited, 162 participants attended (55.5 % men and 45.5 % women, 56.9±8 years and 26.6±4 BMI) (Table 1). This was the final sample for the validation study.

All subjects in this study signed an informed consent. The study was approved by the Ethics Committee for human studies at the Viçosa Federal University.

Biochemical analysis

Blood collection was performed by a trained biochemist, using the vacuum collection technique. Blood samples were stored into the freezer and then taken for analysis. Blood samples analysis to identify A1C concentration were performed on the same day of collection at the Laboratory of Clinical Analysis, Division of Health, Viçosa Federal University, using the HPLC (High Performance Liquid Chromatography) method by the apparatus VARIANT II System (Bio-Rad Laboratories, Inc., USA).

Finnish Diabetes Risk Score (FINDRISC)

Details about the development and validation in a prospective setting and cross-sectional evaluation of the FINDRISC in Finnish population have been published elsewhere [13, 18].

The FINDRISC was produced to be a simple risk calculator that could be conveniently used in primary care and also by individuals themselves, only those variables that were easy to assess without any laboratory tests or those clinical measurements that did not require special skills were included [13].

The final risk score form is a one-page questionnaire containing eight questions, with categorized answers, about age, BMI, waist circumference, physical activity, daily

Table 1 Sample characteristics and results of A1C, estimated average glucose and FINDRISC Score

Variables	Female	Male	Total sample
n	72	90	162
Age (years)	56.5±9.0	57.3±7.2	56.9±8
Weight (kg)	66.2±10.4	74.8±12.1*	71±12.2
Height (m)	1.5±0.05	1.6±0.06*	1.6±0.08
BMI (kg/m^2)	26.8±4.6	26.4±3.5	26.6±4
WC (cm)	92±12.1	94.6±10.8	93.4±11.5
A1C (%)	5.7±0.3	5.7±0.5	5.7±0.4
eAG (mmol/l)	6.6±0.6	6.6±0.8	6.6±0.7
FINDRISC Score	11.3±4.8	11±5.4	11.1±5.1

Data are means ± SD. *Significant difference between gender (P≤ 0.001)

eAG estimated Average Glucose; A1C glycosylated hemoglobin; WC Waist Circumference

consumption of fruits, berries or vegetables, history of antihypertensive drug treatment, history of high blood glucose, and family history of diabetes. These variables predicted diabetes incidence in the original study cohort from which the risk score was developed. Each of the answers to the questions in the form was weighted, corresponding to the risk increase associated to the respective variable in the original model. The total risk score is a simple sum of the individual weight, and values range from 0 to 26 [13].

The FINDRISC was chosen as a tool to be validated in Brazil because it is currently the best screening tool available for use in clinical practice and valid in several countries [8]. Importantly, several authorities such as the European Association for the Study of Diabetes, the European Society of Cardiology, and the International Diabetes Federation Consensus Group have recommended the FINDRISC to be used for the risk stratification purposes in the European population [19, 20].

Statistical methods

The equation proposed by Lwanga and Lemeshow [15] was used for calculating the minimum sample in order to extract a sub-sample for the study of the questionnaire. The comparison of results between men and women was performed through the Mann–Whitney test using the software SigmaPlot for Windows Version 11.0 (Systat Software, Inc., Chicago, IL, USA).

The predictive performance of the risk score was evaluated with respect to the Area Under the Curve (AUC) in a Receiver Operating Characteristics (ROC) curve, sensitivity (the probability of a positive test given to the individual that truly does have the disease), specificity (the probability of a negative test given to the individual that does not have the disease), the Positive Predictive Value—PPV (the probability of the disease giving a positive test), and the Negative Predictive Value—NPV (the probability of a non-diseased giving a negative test).

We realize the ROC curve analysis of all individuals with A1C values ≥ 6.0 and the other only in patients with diabetes (A1C ≥ 6.5 %)) to determine the prevalence of individuals with IGT and diabetes.

These tests were performed using the software MedCalc® Version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium). All tests were calculated using a Confidence Interval (CI) of 95 % and $P \leq 0.05$ was assumed to indicate significance.

Definitions

As indicated by American Diabetes Association [7, 16], we used the A1C test as gold standard for the diagnosis of type 2 diabetes. The A1C test was chosen because it presents some advantages over Oral Glucose Tolerance Test (OGTT) and Fasting Plasma Glucose (FPG) for example: 1) Better index of overall glycemic exposure and risk for long-term complications; 2) Substantially less biologic variability; 3) Substantially less pre-analytic instability 4) Relatively unaffected by acute (e.g. stress or illness related) perturbations in glucose levels [16].

Individuals who had A1C ≤ 5.9 % were considered as "normal", 6.0 % to 6.4 % as "IGT", and A1C ≥ 6.5 % as "with diabetes" [16]. Studies show that the risk of diabetes increase with the increase of A1C and cutoff point ≥ 6.0 % was associated with a highly increased risk of incident diabetes [17]. Therefore, we considered the cutoff point of A1C ≥ 6.0 % as diagnostic of IGT.

Results

When analyzing the data, we divided the sub-sample by gender to ascertain if there were differences in the variables analyzed between men and women. The only existing statistical differences in variables were body weight and height, which showed higher values in men than in women (Table 1). However, these differences did not affect BMI and other variables, which showed no statistical differences (Table 1). Therefore, we consider our sub-sample as being homogeneous with respect to the variables analyzed, and all results presented below are for the total sample.

The prevalence of IGT and diabetes according to the A1C test were 21.6 % and 8.6 % respectively. The Diabetes Risk Score value varied from 1 to 25 and the area under the ROC curve (AUC) by considering all values of A1C ≥ 6.0 % was 0.69 (95 % CI 0.61–0.76) (Fig. 1). The sensitivity, specificity, PPV and NPV by each score in a sample Brazilians are showed in Table 2. Based on the best cutoff point found in this study, we could determine the prevalence of IGT in Brazilians (Table 3).

Discussion

The result of the performance of FINDRISC to identify Brazilians with IGT is satisfactory, indicating that it can be used as a screening tool. The best cutoff point found in Brazilians demonstrates a sensitivity of 75.51 % in identifying people likely to develop diabetes.

Another data that strengthens the use of FINDRISC in the prevention of diabetes is the NPV of 82.4 %, meaning that of the people who submit scores below 9, 82.4 % are not having IGT. Analyzing the data in patients with diabetes (A1C ≥ 6.5 %), resulted in a sensitivity 78.57 % (95 % CI 49.2–95.3 %), specificity of 43.92 % (95 % CI 35.8–

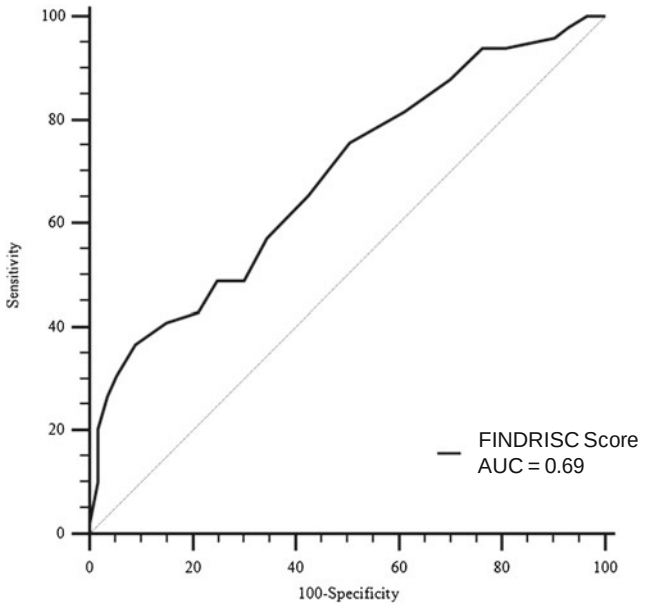

Fig. 1 ROC curves showing the performance of FINDRISC Score for identifying Brazilians with IGT (A1C≥6.0 %)

Table 2 The sensitivity, specificity, PPV and NPV by each score in Brazilians with IGT (A1C≥6.0 %)

Total score	Sensitivity	Specificity	PPV	NPV
1	100.00	0	30.2	100.0
2	100.00	3.54	31.0	100.0
3	97.96	7.08	31.4	88.9
4	95.92	9.73	31.5	84.6
5	93.88	19.47	33.6	88.0
6	93.88	23.89	34.8	90.0
7	87.76	30.09	35.2	85.0
8	81.63	38.94	36.7	83.0
9	75.51	49.56	39.4	82.4
10	65.31	57.52	40.0	79.3
11	57.14	65.49	41.8	77.9
12	48.98	69.91	41.4	76.0
13	48.98	75.22	46.2	77.3
14	42.86	78.76	46.7	76.1
15	40.82	84.96	54.1	76.8
16	36.73	91.15	64.3	76.9
17	30.61	94.69	71.4	75.9
18	26.53	96.46	76.5	75.2
19	20.41	98.23	83.3	74.0
20	10.20	98.23	71.4	71.6
21	6.12	99.12	75.0	70.9
23	2.04	100.00	100.0	70.2
25	0	100.00	100.0	69.8

Data are showed in percentages. AUC 0 0.69 (IC 95 % 0 0.61–0.76) (P ≤ 0.001). Values in bold represent best cutoff point for identifying individuals with IGT

PPV positive predictive value, NPV negative predictive value

Table 3 Prevalence of risk for identifying Brazilians with IGT (A1C≥6.0 %)

Score	Risk classification	% of study sample[a]	% of population[b]
<9	Low	32.8	30.9
≥9	High	67.2	69.1

[a] Study sample from cross-sectional survey

[b] Total population of cross-sectional survey

52.3 %), PPV of 11.7 % (95 % CI 6.0–20 %) and NPV of 95.6 % (95 % CI 87.6–99.1 %).

The present study is the first to validate a screening tool for identify people with risk to IGT in a Brazilian population. We conducted a search in two international databases (Pubmed and Science Direct) until September 2010, and noticed the absence of any study using this type of tool in our population.

Our results are reinforced by recent studies [5, 21] that used questionnaires to identify people with IGT. Schwarz et al. [5] evaluated the usefulness of the FINDRISC to predict insulin resistance in a population with an increasing diabetes risk in a cross-sectional survey (1996) and a cohort study (1997–2000). Although we know that the disease prevalence is different between populations and that this fact influenced the results of the performance analysis of the questionnaires, Schwarz et al. [5] found in the cross-sectional survey (1996) a cutoff point similar to the one found in our study. In the same cutoff point, the results of sensitivity and NPV were quite similar; however, the AUC was different, ours being somewhat lower.

These results provide further evidence for the use of FINDRISC as a tool for screening to identify Brazilian individuals with IGT. The most important use of FINDRISC is the primary health care, where strategies for population-based screening are widely needed. The use by primary care physicians or other health care professionals would facilitate the detection of high-risk subjects and the implementation of early preventive measures.

Current screening tools for type 2 diabetes include questionnaires that assess risk factors, biochemical tests or a combination of both [8]. However, many of these tools require some training prior to their use and invasive biochemical tests, mostly expensive and time consuming. Thus, the need for a simple screening tool based on risk factors for type 2 diabetes, which is self-administered, assumes paramount importance, especially in the community-based settings of developing countries.

It is well known that people with IGT have a high probability to progress into diabetes, approximately half of them will have diabetes within 10 years [22]. The rate of progression to diabetes among people with IGT depends on

their profiles of risk factors, and thus the FINDRISC can be a useful tool to identify those with IGT, which are at greater risks and which would benefit from preventive interventions, as showed recently by the FINDRISC in the Finish Diabetes Prevention Study [23].

The results of this study showed no significance between gender differences (Table 1). Considering these variables as risk factors for diabetes development, the prevalence of gender equality regarding diabetes in Brazil is noteworthy, as has been observed in previous studies [24].

The prevalence of IGT and diabetes according to the A1C test was respectively 21.6 % and 8.6 %. The prevalence of diabetes observed in this study is slightly higher than the estimated prevalence (6.4 %) by International Diabetes Federation (IDF) [2]. However, recent studies done by the Ministry of Health [1] shows that the prevalence of diabetes in Brazil increased from 5.2 % in 2006 to 5.8 % in 2009.

In a recently published study on data from the CARMELA Study [25] was found that the prevalence of diabetes and impaired fasting glucose is high in the seven largest cities in Latin America. Overall, the prevalence of diabetes was 7.0 % (95 % CI 6.5–7.6) and impaired fasting glucose was found in 2 % of the population. However, the data related to the Brazilian population were not included in this study.

When we compared our prevalence data with the CARMELA Study [25], we found that our results are quite close to those found in Mexico City (8.9 % CI 7.7 to 10.2) and Bogota (8.1 %; 95 % CI 6.8 to 9.5). In relation to IGT condition, results of Viçosa city are ten times the ones found in the CARMELA Study [25]. This demonstrates the importance of early diagnosis of people at risk for developing type 2 diabetes, because changes in the lifestyle can prevent or delay the onset of type 2 diabetes in the population [22].

The strength of our study lies in the fact that randomization of the study sample was done and a standard and specific test was used for the diagnosis. The main limitation of our study is the small sample size used to validate the questionnaire. However, the randomization of the study sample potentially decreased the chances of errors. Our sample size calculation was performed by the equation proposed by Lwanga and Lemeshow [15], which is recommended by the World Health Organization (WHO) for epidemiological studies.

In conclusion, our analysis shows that the FINDRISC demonstrated to be a suitable tool to identify Brazilians with IGT.

When implemented in primary health care, the FINDRISC would help health care professionals in decision making regarding the need for further medical investigation and the institution of preventive measures. Furthermore, the application of the FINDRISC in a population-based program also produces a learning effect. People completing the FINDRISC become aware of their own prevalent risk factor.

As proposed by several authorities such as the European Association for the Study of Diabetes, the European Society of Cardiology, and the International Diabetes Federation Consensus Group, the FINDRISC is a useful tool to be used for risk stratification purposes [19, 20]. Brazil, as a developing country of continental dimension, also has a poor health care system, in which a certain percentage of the population lacks access to appropriate health care services and therefore could benefit from using the FINDRISC in primary health care.

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

References

1. Vigitel Brazil 2009: protection and risk factors for chronic diseases by telephone inquiry. 2010. http://portal.saude.gov.br/portal/arquivos/pdf/vigitel2009_220610.pdf. Accessed 2 Oct 2010.
2. International Diabetes Federation - Prevalence estimates of diabetes mellitus (DM), 2010. 2010. http://www.diabetesatlas.org/sites/default/files/DM%202010_7%20regions.xls. Accessed 02 Nov 2010.
3. International Diabetes Federation - Economic impact of Diabetes. 2010. http://www.diabetesatlas.org/sites/default/files/ Economic%20impact%20of%20Diabetes.pdf. Accessed 2 Nov 2010.
4. International Diabetes Federation - Prevalence estimates of diabetes mellitus (DM), 2030. 2010. http://www.diabetesatlas.org/sites/default/files/DM%202030_7%20regions.xls. Accessed 02 Nov 2010.
5. Schwarz PE, Li J, Reimann M, Schutte AE, Bergmann A, Hanefeld M, Bornstein SR, Schulze J, Tuomilehto J, Lindstrom J. The Finnish Diabetes Risk Score is associated with insulin resistance and progression towards type 2 diabetes. J Clin Endocrinol Metab. 2009;94:920–6.
6. Screening for type 2 diabetes. Diabetes Care. 2004;27 Suppl 1:S11–4.
7. Standards of medical care in diabetes—2010. Diabetes Care. 2010; 33 Suppl 1:S11–61.
8. Schwarz PE, Li J, Lindstrom J, Tuomilehto J. Tools for predicting the risk of type 2 diabetes in daily practice. Horm Metab Res. 2008;41:86–97.
9. Gao WG, Qiao Q, Pitkaniemi J, Wild S, Magliano D, Shaw J, Soderberg S, Zimmet P, Chitson P, Knowlessur S, et al. Risk prediction models for the development of diabetes in Mauritian Indians. Diabet Med. 2009;26:996–1002.
10. Gao WG, Dong YH, Pang ZC, Nan HR, Wang SJ, Ren J, Zhang L, Tuomilehto J, Qiao Q. A simple Chinese risk score for undiag- nosed diabetes. Diabet Med. 2010;27:274–81.
11. Cabrera de Leon A, Coello SD, Rodriguez Perez Mdel C, Medina MB, Almeida Gonzalez D, Diaz BB, de Fuentes MM, Aguirre- Jaime A. A simple clinical score for type 2 diabetes mellitus screen- ing in the Canary Islands. Diabetes Res Clin Pract. 2008;80:128–33.

12. Glumer C, Vistisen D, Borch-Johnsen K, Colagiuri S. Risk scores for type 2 diabetes can be applied in some populations but not all. Diabetes Care. 2006;29:410–4.
13. Lindstrom J, Tuomilehto J. The diabetes risk score: a practical tool to predict type 2 diabetes risk. Diabetes Care. 2003;26:725–31.
14. Brazilian Institute of Geography and Statistics. Population census summary. http://www.ibge.gov.br/cidadesat/topwindow.htm. Accessed 2 Apr 2011.
15. Lwanga SK, Lemeshow S. Sample size determination in health studies: a practical manual. Geneva: World Health Organization; 1991.
16. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. Diabetes Care. 2009;32:1327–34.
17. Zhang X, Gregg EW, Williamson DF, Barker LE, Thomas W, Bullard KM, Imperatore G, Williams DE, Albright AL. A1C level and future risk of diabetes: a systematic review. Diabetes Care. 2010;33:1665–73.
18. Saaristo T, Peltonen M, Lindstrom J, Saarikoski L, Sundvall J, Eriksson JG, Tuomilehto J. Cross-sectional evaluation of the Finnish Diabetes Risk Score: a tool to identify undetected type 2 diabetes, abnormal glucose tolerance and metabolic syndrome. Diab Vasc Dis Res. 2005;2:67–72.
19. Schwarz PE, Lindstrom J, Kissimova-Scarbeck K, Szybinski Z, Barengo NC, Peltonen M, Tuomilehto J. The European perspective of type 2 diabetes prevention: diabetes in Europe–prevention using lifestyle, physical activity and nutritional intervention (DE-PLAN) project. Exp Clin Endocrinol Diabetes. 2008;116:167–72.
20. Schwarz PE, Gruhl U, Bornstein SR, Landgraf R, Hall M, Tuomilehto J. The European perspective on diabetes prevention: development and Implementation of A European Guideline and training standards for diabetes prevention (IMAGE). Diab Vasc Dis Res. 2007;4:353–7.
21. Tuomilehto J, Lindstrom J, Hellmich M, Lehmacher W, Westermeier T, Evers T, Bruckner A, Peltonen M, Qiao Q, Chiasson JL. Development and validation of a risk-score model for subjects with impaired glucose tolerance for the assessment of the risk of type 2 diabetes mellitus-The STOP-NIDDM risk-score. Diabetes Res Clin Pract. 2010;87:267–74.
22. Lindstrom J, Ilanne-Parikka P, Peltonen M, Aunola S, Eriksson JG, Hemio K, Hamalainen H, Harkonen P, Keinanen-Kiukaanniemi S, Laakso M, et al. Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study. Lancet. 2006;368:1673–9.
23. Lindstrom J, Peltonen M, Eriksson JG, Aunola S, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukaanniemi S, Uusitupa M, Tuomilehto J. Determinants for the effectiveness of lifestyle intervention in the Finnish Diabetes Prevention Study. Diabetes Care. 2008;31:857–62.
24. Goldenberg P, Schenkman S, Franco LJ. Prevalence of diabetes mellitus: gender differences and sex equalities. Rev Bras Epide- miol. 2003;6:18–28.
25. Escobedo J, Buitron LV, Velasco MF, Ramirez JC, Hernandez R, Macchia A, Pellegrini F, Schargrodsky H, Boissonnet C, Champagne BM. High prevalence of diabetes and impaired fasting glucose in urban Latin America: the CARMELA Study. Diabet Med. 2009;26:864–71.

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ORIGINAL ARTICLE

Efficacy of yoga based life style modification program on medication score and lipid profile in type 2 diabetes—a randomized control study

R. Nagarathna & M. R. Usharani & A. Raghavendra Rao & R. Chaku & R. Kulkarni & H. R. Nagendra

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Abstract Several studies have documented the beneficial short term effects of yoga in type 2 diabetics. In this prospective two-armed interventional randomized control study, 277 type 2 diabetics of both genders aged above 28 years who satisfied the study criteria were recruited from 5 zones in and around Bengaluru, India. They were allocated to a yoga-based life style modification program or exercise-based life style modification program. Integrated yoga special technique for diabetes included yogasanas, pranayama, meditation and lectures on yogic life style. Control intervention included physical exercises and life style education. Medication score, blood glucose, HbA1c and lipid profile were assessed at baseline and after 9 months. Intention to treat analysis showed better reduction ($P < 0.05$, Mann-Whitney test) in the dose of oral hypoglycemic medication required (Yoga - 12.8 %) (Yoga-12.3 %) and increase in HDL (Yoga-7 %) in Yoga as compared to the control group; FBG reduced (7.2 %, P00.016) only in the Yoga group. There was significant reduction within groups ($P < 0.01$) in PPBG (Yoga-14.6 %, Control-9 %), HbA1c (Yoga-14.1 %, Control-0.5 %), Triglycerides (Yoga-15.4 %, Control-16.3 %), VLDL (Yoga-21.5 %, Control-5.2 %) and total cholesterol (Yoga-11.3 %, Control-8.6 %). Thus, Yoga based life style modification program is similar to exercise-based life style modification in reducing blood glucose, HbA1c, triglycerides, total cholesterol and VLDL. Yoga is better than exercise in decreasing oral hypoglycemic medication requirement and LDL; and increasing HDL in type 2 diabetics.

Keywords Yoga · Exercise · Hypoglycemic agent · HDL · Blood glucose

R. Nagarathna (✉)
e-mail: rnagaratna@gmail.com

Introduction

Diabetes mellitus is a major global health problem affecting 150 million people worldwide. In India, the prevalence of type 2 diabetes (T2DM) and premature coronary artery disease is rapidly escalating in all socioeconomic groups parallel with the obesity epidemic [1].

The primary reasons for this rapid global epidemiological transition include aging of the population [2], genetic factors [3], changing life style with altered dietary patterns with decreased physical activity [4], and psychosocial stresses [5]. The associated lipoprotein abnormalities such as elevated concentrations of triglycerides and LDL, with decreased HDL, and the oxidative stress play an important role in the occurrence of early atherosclerosis in diabetics. Hence, the primary role of life style modification programs that include exercise, diet and stress reduction has been widely accepted to reduce the incidence of type 2 diabetes [6, 7, 8]. There are reports that the physician's advice in a diabetic clinic is usually ineffective [8]. Studies have also shown that people have considerable interest in lifestyle interventions than a pharmaceutical trial [9]. Incidence of type 2 diabetes has reduced by 40 to 60 % over 3 to 4 years in high risk population in USA after modest weight loss through diet and physical activity [7].

Alternative methods of exercise, stress reduction [10], and relaxation techniques [11] including cognitive behaviour therapy [12] have been shown to improve the mood with better glycemic control and prevention of complications of the metabolic syndrome. Psychological stresses resulting in depression contributes to poor compliance and outcome of therapeutic measures [6] and its treatment has shown better glycemic control and improved quality of life [13]. Yoga has been explored scientifically since the 1970's as a widely available resource for life style-related problems such as hypertension [14], bronchial asthma [15], diabetes [16] and coronary artery disease [17]. A critical review of all published literature from 1970 to 2006 on the effects of yoga based programs on the risk profiles in adults with T2DM showed that yoga reduces the risk profiles and may help in prevention and management of its cardiovascular complications [18]. These beneficial effects of yoga seem to be due to the relaxation response [16] that has the potential to reduce the heightened stress responses through techniques that promote mastery over the modifications of mind [19].

Studies in India have also shown the beneficial effects of yoga in diabetics. Damodaran et al observed decrease in blood pressure, drug scores, sympathetic activity (VMA catecholamine and MDA), oxidant stress (vitamin C cholinesterase) and improvements in risk factors such as blood glucose, cholesterol and triglycerides with better subjective well being and quality of life in a non randomized study on outpatients for 3 months [20]. Singh et al showed significant decrease in fasting and post prandial blood glucose levels and glycosylated hemoglobin with stable autonomic functions after forty days of yogic exercises in Type 2 Diabetics [21].

More recently, Hegde et al (2011) in south India in a stratified control trial studied diabetics with and without complications (peripheral neuropathy or microvascular or macrovascular) and observed significant reduction in BMI, improved glycemic control, and oxidative stress within 3 months when yoga was added to standard care [22]. Yoga improved the 'heart friendly' status of lipid profile in peri and post-menopausal patients receiving standard medical treatment for type 2 DM with decrease in low density lipoprotein as well as fasting and postprandial blood glucose levels within six weeks [23]. A review by the American diabetes association on yoga and other mind body intervention concluded that clinical trials on patients with diabetes have shown improvement in measures of quality of life and stress but consistent long-term improvements in glycemic control or HbA1C have not been documented [24].

There are not many long-term follow up studies which have directly compared the effects of exercise with yoga on medication requirement and lipid profile. Hence, the present study was planned with an objective to compare the efficacy of a Yoga based Life Style modification Program (YLSP) with conventional Exercise based Life Style modification Program (ELSP) in type 2 diabetics, with the hypothesis that YLSP will be better than ELSP, in achieving better control of diabetes with favourable changes in medication requirement and lipid profile.

Subjects and methods

Design

The study Registered Trial number - CTRI/2008/091/000293 was a prospective randomized two arm parallel control study with active intervention for the control group. The research protocol was approved by the Ethics Committee of SVYASA University. Signed informed consent was obtained from all participants before recruitment.

Subjects

The participants were selected from five different zones (east, west, north, south and central) in and around Bengaluru city, India, between 2003–2007. Inclusion criteria were, (a) type 2 diabetics of both sexes above 25 years, (b) fasting venous blood glucose level >120 mg% at the time of diagnosis (checked from their records), (c) T2DM of more than one year, (d) those stabilized on a stable dosage of oral hypoglycemic agents or insulin for at least three weeks and (e) no prior exposure to yoga practice. Those with major complications of T2DM such as chronic infections (tuberculosis, HIV), coronary artery disease, severe hypertension, nephropathy, proliferative retinopathy and/or cerebrovascular disease were excluded. Those with peripheral neuropathy, mild urinary or cutaneous fungal infections, mild to moderate

hypertension and obesity were not excluded. The sample size was calculated by Cohen's formula [25] with alpha of 0.05, powered at 0.80 for an effect size of 0.34 based on our earlier interventional study [16]. All participants who satisfied the selection criteria and signed the informed consent were assigned a numbered envelope containing a computer generated random number (using www.randomizer.org). To conceal bias during randomization, the statistician at the university centre, who was not involved in administering the intervention, allocated them to yoga or control groups. Different random number tables were used for five different venues.

Masking

As this was an interventional study blinding was not possible. The laboratory staff and the statistician were blind to the source of the data. Care was taken to prevent crossover of participants or interaction between the two groups.

Methods

Within one week after recruitment all baseline data were documented. Those who needed any change in the dosage of oral hypoglycemic medication or insulin were made to wait for 3 weeks for a second assessment before starting the intervention. Both groups were trained by certified therapists through daily (one hour/day - 5 days /week) classes, for 12 weeks followed by weekly follow up classes for 2 h for 9 months. During the follow up period they were asked to continue the practices for 1 h daily at home using a pre-recorded instruction audio tape. All participants were provided with a diary to tick the type and duration of their daily recommended practice, monthly fasting and post-prandial blood glucose levels, body weight, visits to family physician for any health problem, episodes of hypoglycemia, and any change in their diet, medication or daily practice of yoga or exercise. The medical officer and the dietitian were available at the venue once a month to check their progress and advise suitable changes. All outcome measures for the study were checked at the end of 9 months.

Intervention

The modules of intervention for the two groups were carefully developed by a team consisting of two senior yoga experts of the university, a psychiatrist and a diabetologist. Table 1 shows the details of the intervention for the two groups. The interventions for both modules were aimed at achieving, (a) conventional diabetes education, (b) stress management, and (c) empowerment for adherence to long term life style change. The specific yoga module for YLSP was the same as that used in our earlier study on yoga in diabetes [16]. This 'Integrated Approach of Yoga for Diabetes (IAYD)' is based on the knowledge culled out from yoga scriptures (Patanjali yoga sutras, Bhagvadgita and Mandukya karika).

The practices included (i) physical practices such as cleansing techniques (kriyas), loosening practices (shithilikarana vyayama), sun salutation (suryanamaskara) and yoga postures (asanas) to provide mild intensity physical exercise effect. (ii) Pranayama and meditation (dharana and dhyana) for calmness of mind, (iii) devotional sessions (Bhakti yoga) for better emotional stability and (iv) lectures and yogic counseling for notional correction through self analysis (Jnana yoga) [16, 26]. The kriyas (Neti, Dhouti and Shankaprakshalana) were done once a week. The ELSP module consisted of (i) standard physical training (PT) exercises and walking designed to achieve a comparable intensity of physical exertion, (ii) non-yogic breathing exercises used in physiotherapy and (iii) supine rest. Both groups had access to reading material on conventional diabetes education.

Measurements

Baseline measurements before recruitment included demographic data and investigations to satisfy the selection criteria. They were (i) fasting blood glucose, (ii) resting blood pressure using a sphygmomanometer, (iii) electrocardiogram using a portable ECG recorder (one channel recorder, version 6108 T, BPL, India), (iv) fundoscopy by a certified ophthalmologist and (v) serum urea and creatinine [27] to look for nephropathy.

Outcome measures

A semi-structured interview for medical history, and demographic data were recorded by the medical officer after recruitment.

1. Medication score: The oral medication scores (standard quantity of the drug per tablet as indicated in CIMS India [28] expressed as number of tablets per day) were calculated separately for each category i.e. Oral Hypoglycemic Agents (OHA), Lipid Lowering Drugs (LLD) and Antihypertensive drugs (AHT). Total medication score indicates the total number of tablets of all drugs consumed in a day. The insulin score was calculated by using a scoring system ranging from 0–3 (0 0 nil, 1 e 15 units, 2 0 16–30 units, 3 > 30 units), for the total number of units of insulin injected in 24 h.
2. Biochemical measures included blood glucose, HbA1c and lipid profile. Blood samples were drawn from an antecubital vein in the fasting state (Fasting Blood Glucose—FBG) between 8 am and 9 am, and 2 h after breakfast (Post-Prandial Blood Glucose—PPBG) between 10 am and 11 am. The participants were instructed to abstain from morning yoga or PT exercises on the day of blood

Table 1 Practices used for the intervention in both groups

Practices for Yoga (YLSP) group	Practices for control (ELSP) group
Breathing Exercises	Exercises in standing position
Shasha Shwasa (Rabbit Breathing)	Forward backward bending
Vyaghra Shwasa(Tiger Breathing)	Side bending
Navasana Shwasa (Boat Breathing)	Jogging
Shithilikarana Vyayama (yogic loosening)	Sit-up
Padahatasana Ardha Cakrasana Chalana (Forward Backward Bending)	Twisting
	Cross leg lifting
Trikonasana Chalana (Side Bending)	Alternative toe touching
Kati Parivartana Chalana (Twisting)	Hip rotation
Dhanurasana Chalana (Swinging in Bow)	Knee rotation
Pavanamuktasana Kriya (wind releasing)	forward drill
Surya Namaskara (Sun Salutation)	Backward drill
Shavaansa (Quick Relaxation Technique—QRT)	Sideward drill
Asanas (Yoga postures)	Neck movements
Parivritta Trikonasana (Twisted triangle)	Shoulder rotation
Vakrasana or Ardha Matsyendrasana (Spinal Twist or Sage Matsyendra posture)	Full arm rotation
Ustrasana (Camel)	Free walking
Hamsasana or Mayurasana (Swan or Peacock)	Exercises in sitting position
Bhujangasana (Cobra)	Knee cap tightening
Dhanurasana (Bow)	Swimming
Sarvangasana (Whole Body Inverted posture)	Supine relaxation
Matsyasana (Fish)	Half butterfly exercise
Deep Relaxation Technique (DRT)	Chakki chalana (waist twisting))
Bandhas (locks) and Kriyas (cleansing)	Ankle bend exercise
Jala Neti (nasal wash with water)	Toe Bend exercise
Sutra Neti (nasal wash with catheter)	Crow walking
Vaman Dhouti (yogic vomiting)	Knee rotation
Shankha Prakshalana(yogic bowel cleansing)	Exercises in prone position
Uddiyana Bandha (diaphragm lock)	Prone bow swing
Agnisara Kriya(abdominal flap)	Prone Alternate
Kapalabhati (Blasting breath)	head and leg swing
Pranayama (yogic breathing)	Boating
Vibhagiya Pranayama (Sectional Breathing)	Rolling
ujjayi (glottis breathing)	Alternate Arm swing
Nadi Suddhi (alternate nostril)	Hip stretch
Sitali or Sitkari (cooling breath)	Exercises in Supine position.
Bhramari (Bee Breathing)	Cycling
Meditation (Dharana and Dhaya)	Straight leg rising
Nadanusandhana (sound resonance Merger)	Side leg rising
Om Meditation (meditation on Om syllable)	Knee exercise
Lectures—topics covered	Dorsal Stretch
Diabetes: burden, causes, management	Rolling and rocking
Yogic concepts of healthy life style	Supine rest
including thinking, feeling and behaviour,	Lectures—topics covered
Yogic management of stress,	Diabetes: burden, cause, management causes, management.
Diabetes and yoga Diet,	Modern scientific concepts of healthy life style including thinking, feeling and behaviour
How to stop smoking, the yogic way	Modern concepts of management of stress and how to stop smoking

YLSP Yoga based Life Style change Program, ELSP Exercise based Life Style change Program

collection. The sera were separated within an hour of collection. A certified technician carried out all the tests at the SVYASA university laboratory. Heparinised blood was used to analyze HbA1c by using affinity assay method on a Nycocord reader [29]. The concentration of glucose was determined by using glucose-oxidase method [30] and serum total cholesterol, triglyceride and HDL by enzymatic methods [31]. High density lipoprotein was measured after precipitating VLDL and LDL cholesterol in the presence of magnesium ions. The VLDL and LDL fractions were calculated by the Friedwald's formula [32].

Statistical analysis

Data were analyzed using SPSS version 16. The baseline data were not normally distributed (Shapiro Wilk's test $P<0.05$). Mann Whitney test was used for pre values for checking the baseline matching. As there was an attrition rate of about 38 % by the 9th month, we carried out 'intention to treat analysis' considering both pre and post data as predictors [33–35] based on the concept of 'Expectation Maximisation'. Wilcoxon's signed ranks test was used to compare the pre-post changes and Mann-Whitney 'U' test to compare groups.

Results

Figure 1 shows the trial profile. Out of 520 screened, 277 (87 females) participants (141 in YLSP and 136 in ELSP), were randomized into two groups; 264 completed the initial 12 weeks of training and 173 (88 in yoga and 85 in control group) completed the study. The reasons for drop outs are given in Fig. 1. Table 2 shows the demographic features. There was no baseline matching between groups in mean duration of diabetes before recruitment (6.19±5.49 years in YLSP and 4.75±4.18 years in ELSP). The baseline measurements used to rule out nephropathy before recruitment was matched for creatinine concentration and blood urea nitrogen in both groups.

Medication score (Table 3)

Oral hypoglycemic drug requirement reduced in 30 participants in YLSP and 14 in ELSP with significant reduction of mean scores in YLSP (12.8 %, $P<0.001$) and non-significant reduction (3.7 %) in ELSP. There was significant difference between groups at P00.05 (Mann Whitney). The total medication that included all categories of drugs reduced by 10.9 % in YLSP (P00.004) with no significant difference in ELSP or between groups. It reduced in 35 patients in YLSP and 19 patients in ELSP. Amongst those who were taking insulin (16 in YLSP and 10 in ELSP) at the time of recruitment, five in YLSP group and one in ELSP had discontinued (no significant statistical change).

Lipid profile (Table 4)

HDL increased by 7 % in YLSP (P00.002) with significant difference between groups (P00.007). LDL reduced significantly in YLSP by 12.3 % ($P<0.001$), with difference between groups at P00.003. Triglycerides, total cholesterol and VLDL reduced significantly in both groups with non significant differences between groups and better effect sizes in yoga group.

Changes in blood glucose (Table 4)

There was a significant reduction in FBG by 7.2 % in YLSP (P00.016) at 9th month. PPBG reduced significantly in both the groups, 14.6 % in YLSP ($P<0.001$) and 8.9 % in ELSP (P00.019) groups, with non-significant difference between groups. The concentration of HbA1c reduced in both groups, 14.1 % in YLSP ($P<0.001$) and 0.5 % in ELSP (P00.002) with no significant difference between groups.

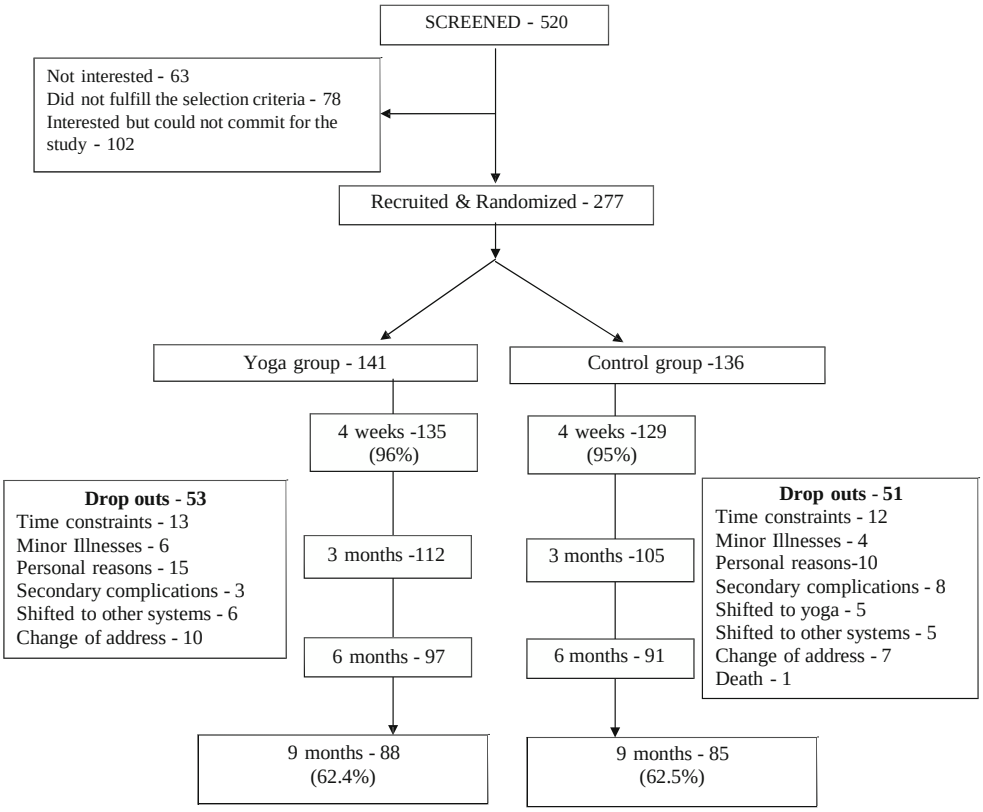
Subgroup analysis based on duration of illness:

A subgroup analysis was done between groups with a median cut off of 5 years for duration of illness. The trends observed between groups when duration of illness was <5 years or >5 years was similar to trends seen when groups were compared without cut offs for duration of illnesses as reported above. Hence, this has not been reported separately in tables.

Discussion

This was a prospective randomized control study that compared YLSP with ELSP on 277 participants with type 2 diabetes selected from 5 zones in and around Bengaluru. After 9 months of intervention there was significant difference between groups ($P<0.01$) in HDL, LDL and medication requirement with higher effect sizes in YLSP group. There was reduction in PPBG and HbA1c, triglycerides, total cholesterol and VLDL in both groups with better effect sizes in yoga group (non-significant differences between groups) whereas FBG reduced significantly only in the yoga group. A review by Innes KE et al [18] of 25 yoga studies on type 2 diabetics (of which 4 were RCTs), concluded that yoga practice was associated with reduction of 6.1–34.4 %

Fig. 1 Trial profile



in blood glucose and 10.5–27.3 % in HbA1c. A recent three armed RCT showed a 30 % reduction in FBG with no changes in HbA1c or medication scores after 6 months of yoga [36]. Our study adds evidence to the efficacy of yoga in a south Indian diabetic population. This is the first study that has documented significantly better reduction in oral hypoglycemic agents (12.8 %) in YLSP than ELSP.

Significant reduction in serum total cholesterol, triglycerides and LDL concentrations in type 2 diabetics after yoga exercises [37] and Sudarshan Kriya Yoga (SKY) [38] have

Table 2 Demographic data

	Variables		YLSP group (141)	ELSP group (136)
1	Gender	Males	91	99
		Females	50	37
2	Age	Range	30–78 years	30–74 years
		Mean±SD	53.46±8.86	51.38±8.39
3	Education	School	78	73
		Undergraduates	36	38
		Graduates	27	22
		Post Graduates	–	3
4	Socio-Economic Status	Upper class	1	0
		Middle class	140	136
5	Duration of DM	Mean±SD	6.19±5.49	4.75±4.18
		1–5	92	96
		5.1–10	25	29
		10–20	20	9
		20–30	2	1
		30–40	2	1
6	Family History of DM2		63	45
7	Tobacco chewing		19	9
8	Alcohol Consumption		13	12

YLSP Yoga based Life Style change Program, ELSP Exercise based Life Style change Programs

Table 3 Results- Changes in medication scores after 9 months of intervention in both groups

Variable	Group	Pre-intervention	Post-intervention	% Change	Within groups		Between groups	
		Mean ± SD	Mean ± SD		P	ES	P	ES
Total	YLSP (N0 141)	3.38 ± 2.19	3.01 ± 1.88	10.94	0.004	0.29	NS	–
	ELSP (N0 136)	3.24 ± 1.95	3.15 ± 1.74	2.77	0.37	0.09		
OHA	YLSP	2.27 ± 1.29	1.98 ± 1.27	12.77	<0.001	0.37	0.05	0.25
	ELSP	2.41 ± 1.52	2.32 ± 1.42	3.65	0.22	0.11	NS	–
LLD	YLSP	0.76 ± 0.44	0.88 ± 0.48	15.79	NS	–	NS	–
	ELSP	0.77 ± 0.43	0.92 ± 0.28	19.48	NS			
Anti HT	YLSP	1.10 ± 0.52	1.08 ± 0.47	1.81	NS	–	NS	–
	ELSP	0.98 ± 0.68	1.02 ± 0.51	4.08	NS			

OHA Oral Hypoglycemic Agents, LLD Lipid Lowering Drugs, Anti HT Antihypertensive drugs, ES Effect Size, NS Not Significant

Table 4 Changes in glycemic control and lipids in both groups after 9 months of intervention

Variables		YLSP (N0 141)				ELSP (N0 136)				Between Groups	
		Mean ± SD	95% CI	pre-post		Mean ± SD	95% CI	pre-post		ES	P Post YLSP vs Post ELSP
			LB UB	% ES	P		LB UB	% ES	P		
FBG	Pre	133.72 ± 44.52	126.65 140.78	7.2%	0.016	130.31 ± 39.58	123.21 137.40	3.9 %	NS	0.03	0.53
	Post	124.08 ± 32.46	118.93 129.23	0.22		125.20 ± 32.65	119.34 131.05	0.12			
PPBG	Pre	184.02 ± 72.53	172.51 195.53	14.6%	<0.001	171.90 ± 65.25	160.21 183.60	8.9%	0.019	0.01	0.72
	Post	157.19 ± 55.22	148.43 165.95	0.36		156.48 ± 55.01	146.62 166.34	0.21			
HbA1c	Pre	8.54 ± 1.68	8.27 8.80	14.1%	<0.001	8.07 ± 1.42	7.81 8.32	0.5%	0.002	0.19	0.11
	Post	7.33 ± 3.00	6.86 7.81	0.38		8.03 ± 4.26	7.27 8.80	0.01			
HDL	Pre	44.75 ± 13.82	42.56 46.95	7%	0.002	45.11 ± 13.43	42.70 47.52	2.1%	NS	0.32	0.007
	Post	47.88 ± 11.80	46.01 49.75	0.20		44.16 ± 11.51	42.10 46.22	0.06			
LDL	Pre	91.16 ± 33.10	85.90 96.41	12.3%	0.001	92.59 ± 35.26	86.27 98.91	0.9%	NS	0.38	0.003
	Post	79.89 ± 30.20	75.10 84.68	0.28		91.72 ± 31.93	86.00 97.45	0.02			
Trigly	Pre	174.10 ± 81.22	161.22 186.99	15.4%	<0.001	180.86 ± 102.59	162.47 199.25	16.3%	0.018	0.08	0.64
	Post	147.28 ± 49.14	139.49 155.08	0.31		151.38 ± 51.66	142.12 160.64	0.28			
T.Cho	Pre	182.86 ± 39.55	176.58 189.13	11.3%	<0.001	182.09 ± 41.31	174.69 189.50	8.6%	<0.001	0.11	0.38
	Post	162.20 ± 36.74	156.37 168.03	0.47		166.34 ± 37.94	159.54 173.14	0.32			
VLDL	Pre	44.30 ± 23.26	40.61 47.99	21.5%	<0.001	42.33 ± 17.71	39.16 45.50	5.2%	0.009	0.23	0.18
	Post	34.76 ± 12.12	32.84 36.69	0.38		40.13 ± 31.14	34.55 45.71	0.06			

YLSP Yoga based Life Style change Program, ELSP Exercise based Life Style change Program, ES Effect Size, FBG Fasting Blood Glucose, PPBG Post Prandial Blood Glucose, HbA1c Glycosylated Hemoglobin, HDL High Density Lipoprotein, LDL Low Density Lipoprotein, Trigly Triglycerides, T.chol Total Cholesterol, VLDL Very Low Density Lipoprotein

been observed. The yoga exercise study [37] showed an increase in HDL whereas this was not achieved after SKY. Significant improvement in HDL and LDL profiles in YLSP group in the present study with an increase in HDL (7 %) in the yoga group is noteworthy.

Several studies have proven the efficacy of different types of exercises in increasing HDL and decreasing LDL [6, 10–12, 39]. It appears that moderate intensity exercises (and not vigorous intensity exercises) are effective in reducing VLDL complex of triglycerides, whereas sustained increase in HDL may occur only after vigorous exercises such as jogging [39]. This may explain the non-significant changes in HDL and LDL in our ELSP group. This also seems to indicate that the increase in HDL found in the YLSP group may involve pathways other than its exercise component. Activation of hypothalamic pituitary axis (HPA) axis and sympatho-adrenal system is known to inhibit glucose uptake by peripheral tissues by inhibiting insulin release, inducing insulin resistance and increasing hepatic glucose production [40]. Better sympathovagal balance [16, 41] better insulin receptor sensitivity [42, 43] and reduced oxidative stress [44] may have contributed to the beneficial effects of the integrated yoga practices.

Major strengths of this study includes, the longitudinal prospective randomized multi-venue control design with good sample size selected from five zones of a metropolitan city in south India, active intervention for the control group, follow up duration of nine months and the results showing significant reduction in oral hypoglycemic medication better than control group. Limitations of this study were: (a) the data of body weight, BMI and calorie intake before and after the intervention could not be reported because different instruments were used in different venues. These parameters were documented in the diaries and used for advice during the monthly medical monitoring; (b) the compliance for the initial classes of 12 weeks was good with 95 % attendance with an attrition rate of 38 % by 9^{th} month. This attrition rate is similar to that reported in clinical trials involving diabetics for self monitoring and management of diabetes (2.3 % – 50 %) [33]. This attrition rate could be attributed to longer duration of intervention. The yoga based lifestyle program was safe and did not cause any injuries to participants. All facets of the yoga program were equally adhered to by the participants.

In conclusion, YLSP is better than ELSP in reducing the requirement of oral hypoglycemic agents, increasing HDL and decreasing LDL and YLSP is similar to ELSP in reducing blood glucose, HbA1c, triglycerides, VLDL and total cholesterol levels. Our study suggests that yoga, a non-expensive technique that has become popular around the globe with good acceptability and generalizability, may be incorporated in all primary and secondary prevention programs for type 2 diabetics in clinical practice.

Future research should be three armed randomised control designs to control for other confounding variables such as diet, weight and monitoring of VO^2 max to match the intensity of exercises between groups and inclusion of other measures to understand the underlying mechanisms.

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References

1. Mohan V, Jaydip R, Deepa R. Type 2 diabetes in Asian Indian youth. Pediatr Diabetes. 2007;8 Suppl 9:28–34.
2. Winer N, Sowers JR. Diabetes and arterial stiffening. Adv Cardiol. 2007;44:245–51.
3. Ramachandran A, Snehalatha C, Sivasankari S, Hitman GA, Vijay V. Parental influence on the spectrum of type 2 diabetes in the offspring among Indians. J Assoc Physicians India. 2007;55:560–2.
4. Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes: Indian scenario. Indian J Med Res. 2007;125:217–30.
5. Chandola T, Brunner E, Marmot M. Chronic stress at work and the metabolic syndrome: prospective study. BMJ. 2006;332:521–5.
6. Gross R, Olfson M, Gameroff MJ, Carasquillo O, Shea S, Feder A, et al. Depression and glycemic control in Hispanic primary care patients with diabetes. J Gen Intern Med. 2005;20:460–6.
7. Williamson DF, Vinicor F, Bowman BA. Primary prevention of type 2 diabetes mellitus by lifestyle intervention: implications for health policy. Ann Intern Med. 2004;140:951–7.
8. Egede LE. Lifestyle modification to improve blood pressure control in individuals with diabetes: is physician advice effective? Diabetes Care. 2003;26:602–7.
9. Sigal RJ, Kenny GP, Boule NG, Wells GA. Prud'homme D, Fortier M, et al. Effects of aerobic training, resistance training, or both on glycemic control in type 2 diabetes: a randomized trial. Ann Intern Med. 2007;147:357–69.
10. Surwit RS, van Tilburg MA, Zucker N, McCaskill CC, Parekh P, Feinglos MN, et al. Stress management improves long-term glycemic control in type 2 diabetes. Diabetes Care. 2002;25:30–4.
11. Surwit RS, Feinglos MN. The effects of relaxations on glucose tolerance in non-insulindependent diabetes. Diabetes Care. 1983;6:176–9.
12. Lustman PJ, Griffith LS, Freedland KE, Kissel SS, Clouse RE. Cognitive behavior therapy for depression in type 2 diabetes mellitus. A randomized, controlled trial. Ann Intern Med. 1998;129:613–21.
13. Delamater AM, Jacobson AM, Anderson B, Cox D, Fisher L, Lustman P. et al. Psychosocial therapies in diabetes: report of the Psychosocial Therapies Working Group. Diabetes Care. 2001;24:1286–92.
14. Sundar S, Agrawal SK, Singh VP, Bhattacharya SK, Udupa KN, Vaish SK. Role of yoga in management of essential hypertension. Acta Cardiol. 1984;39:203–8.
15. Nagarathna R, Nagendra HR. Yoga for bronchial asthma: a controlled study. Br Med J (Clin Res Ed). 1985;291:1077–9.

16. Monro R, Powar J, Anil C, Nagarathna R, Dandona P. Yoga therapy for NIDDM- a control trial. J Complemen Med Res.
1992;6:66–8.
17. Manchanda SC, Narang R. Yoga and coronary artery disease. Indian Heart J. 1998;50:227–8.
18. Innes KE, Vincent HK. The influence of yoga-based programs on risk profiles in adults with type 2 diabetes mellitus: a systematic review. Evid Based Complement Alternat Med. 2007;4:469–86.
19. Nagarathna R, Nagendra HR, Monro R. Yoga for common ailments. 3rd ed. London: GAIA; 1991.
20. Damodaran A, Malathi A, Patil N, Shah N, Suryavansihi, Marathe S. Therapeutic potential of yoga practices in modifying cardiovascular risk profile in middle aged men and women. J Assoc Physicians India. 2002;50:633–40.
21. Singh S, Malhotra V, Singh KP, Madhu SV, Tandon OP. Role of yoga in modifying certain cardiovascular functions in type 2 diabetic patients. J Assoc Physicians India. 2004;52:203–6.
22. Hegde SV, Adhikari P, Kotian S, Pinto VJ, D'Souza S, D'Souza V. Effect of 3-month yoga on oxidative stress in type 2 diabetes with or without complications: a controlled clinical trial. Diabetes Care. 2011;34:2208–10.
23. Madanmohan, Bhavanani AB, Dayanidy G, Sanjay Z, Basavaraddi IV. Effect of yoga therapy on reaction time, biochemical parameters and wellness score of peri and post-menopausal diabetic patients. Int J Yoga. 2012;5:10–5.
24. Birdee GS, Yeh G. Complementary and Alternative Medicine Therapies for Diabetes: A Clinical Review. Clinical Diabetes. 2010;28:147–55.
25. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39:175–91.
26. Rai L, Ram K, Kant U, Madan SK, Sharma SK. Energy expenditure and ventilatory responses during Siddhasana–a yogic seated posture. Indian J Physiol Pharmacol. 1994;38:29–33.
27. Schreiber WE, Busser JR, Huebsch S. Which laboratory tests do students in an internal medicine clerkship need to learn about? Am J Clin Pathol. 2008;130:696–701.
28. Current Index of Medical Specialties (CIMS). India:CMP Medica, 2007 http://www.mims.com/drug/dose. Accessed 17 July 2011.
29. St John A, Davis TM, Goodall I, Townsend MA, Price CP. Nurse-based evaluation of point-of care assays for glycated haemoglobin. Clin Chim Acta. 2006;365:257–63.
30. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin
Chem. 1982;28:2077–80.
31. Lindgren FT, Silvers A, Jutaglr R, Layshot L, Bradley DD. A comparison of simplified methods for lipoprotein quantification using the analytic ultracentrifuge as a standard. Lipids. 1977;12:278–
82.
32. Yagi K. Lipid peroxides and human diseases. Chem Phys Lipids. 1987;45:337–51.
33. Heneghan C, Perera R, Ward AA, Fitzmaurice D, Meats E, Glasziou P. Assessing differential attrition in clinical trials: self-monitoring of oral anticoagulation and type II diabetes. BMC Med Res Methodol. 2007;7:18.
34. Schafer JL, Graham JW. Missing data: our view of the state of the art. Psychol Methods. 2002;7:147–77.
35. Dempster AP, Laird NM, Rubin DB. Maximum likelihood from incomplete data via the EM algorithm. J Royal Stat Soc, Ser B. 1977;39:1–38.
36. Gordon LA, Morrison EY, McGrowder DA, Young R, Fraser YT, Zamora EM, et al. Effect of exercise therapy on lipid profile and oxidative stress indicators in patients with type 2 diabetes. BMC Complement Altern Med. 2008;8:21.
37. Agrawal R, Aradhana R, Hussain S, Sabir M, Kochar D, Kothari R. Influence of yogic treatment on quality of life outcomes, glycaemic control and risk factors in diabetes mellitus. Int J Diabet Dev Ctries. 2003;23:130–4.
38. Agte VV, Tarwadi K. Sudarshan kriya yoga for treating type 2 diabetes: a preliminary study. Altern Complet Therap. 2004;10:220–2.
39. Iborra RT, Ribeiro IC, Neves MQ, Charf AM, Lottenberg SA, Negrao CE, et al. Aerobic exercise training improves the role of high-density lipoprotein antioxidant and reduces plasma lipid peroxidation in type 2 diabetes mellitus. Scand J Med Sci Sports.
2008;18:742–50.
40. Fehm HL, Kern W, Peters A. The selfish brain: competition for energy resources. Prog Brain Res. 2006;153:129–40.
41. Vempati RP, Telles S. Yoga-based guided relaxation reduces sympathetic activity judged from baseline levels. Psychol Rep.
2002;90:487–94.
42. Chaya MS, Ramakrishnan G, Shastry S, Kishore RP, Nagendra H, Nagarathna R, et al. Insulin sensitivity and cardiac autonomic function in young male practitioners of yoga. Natl Med J India.
2008;21:217–21.
43. Timmermans RJ, Saris WH, van Loon LJ. Insulin resistance: the role of intramuscular triglyceride and the importance of physical activity. Ned Tijdschr Geneeskd. 2006;150:122–7.
44. Singh S, Malhotra V, Singh K, Sharma S. A preliminary report on the role of yoga asanas on oxidative stress in non-insulin dependant diabetes. Indian J Clin Biochem. 2001;16:216–20.

ORIGINAL ARTICLE

Validation of a screening tool for identifying Brazilians with impaired glucose tolerance

Bruno Pereira de Moura & Paulo Roberto do Santos Amorim & Sylvia do Carmo Castro Franceschini & Janice Sepúlveda Reis & João Carlos Bouzas Marins

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Abstract This study was designed to validate a self-assessment tool for identifying Brazilians with Impaired Glucose Tolerance (IGT). The Finnish Diabetes Risk Score (FINDRISC) was applied in 829 people aged over 40 years without previous diagnosis of diabetes between November 2009 and April 2010 at the Viçosa city, Minas Gerais, Brazil. We randomly selected 300 subjects in the initial survey, which received by post a letter of invitation to attend the laboratory for the collection of the blood sample to identify levels of glycated hemoglobin (A1C). Of these 300 subjects invited, 162 attended subjects, this being the final sample for the validation study. The risk score was evaluated through the Area Under the Curve (AUC) in a Receiver Operating Characteristics (ROC) curve. We assume a maximum error of 5 % and confidence interval 95 %. The prevalence of IGT and diabetes according to the A1C test were 21.6 % and 8.6 % respectively. The Diabetes Risk Score value varied from 1 to 25. The AUC by considering all values of A1C ≥ 6.0 % was 0.69 (95 % CI 0.61–0.76). The score value ≥ 9 had sensitivity of 75.51 %, specificity of 49.56 %, Positive Predictive Value (PPV) of 39.4 % and Negative Predictive Value (NPV) of 82.4 %. The FINDRISC is a suitable tool to identify Brazilians with IGT.

Keywords Type 2 diabetes · Screening · Impaired glucose tolerance · Assessment tool

P. R. S. Amorim (*)
e-mail: pramorim@ufv.br

Introduction

A recent study by the Ministry of Health [1] shows that the prevalence of diabetes in Brazil increased from 5.2 % in 2006 to 5.8 % in 2009. According to International Diabetes Federation (IDF) in 2010 [2], the estimated prevalence was 6.4 %, meaning 7,632,500 people with diabetes across the country and placing Brazil as the fifth country with the largest number of people with diabetes [3]. In 2030, it is estimated that the prevalence will rise to 7.7 % of the population [4].

The growing number of people with diabetes in their productive age increases the economic burden on the health care system, due to increasingly early onset of complications and subsequently a period of intensive long medical treatment [5].

Based on this evidence, identifying individuals with high risk for developing diabetes (IGT) or those already with the disease is extremely important [6]. However, the American Diabetes Association (ADA) [7] recommends that the tests to detect type 2 diabetes and assess risk for future diabetes in asymptomatic people should only be considered in adults with some additional risk factors.

Thus, in order to identify individuals at high risk of developing type 2 diabetes several simple, inexpensive and non-invasive tools were developed and tested in many populations over the last decade [8–11]. Such tools cannot be applied to other ethnic groups, except when validated for the targeted population [12]. Such predicting tools are lacking in the Brazilians populations. Therefore, the current study was conducted to validate a self-assessment tool for identifying Brazilians with IGT.

Participants and methods

Methods

This cross-sectional survey was conducted between November 2009 and April 2010 at the Viçosa city, Minas Gerais. The questionnaire to screen for diabetes was applied in 829 individuals (≥ 40 years) without previous diagnosis of diabetes. Questionnaires were administered by health workers in 15 units of the Family Health Program of the city and seven points at the university campus. Health workers involved in collecting data were trained and the equipment used for measuring body weight and height of the subjects had been tested and calibrated. Waist circumference was measured at the navel, according to the instructions of the questionnaire [13].

Subjects

The Viçosa city has about 72,220 inhabitants (51.5 % women). Approximately 26,133 (36.2 %) people are more than 40 years of age [14]. Currently, according to International Diabetes Federation (IDF) [2], the estimated prevalence of diabetes in Brazil is 6.4 %. Based on this information, we used the equation proposed by Lwanga and Lemeshow [15] for calculating the minimum sample in order to extract a sub-sample for the study of the questionnaire. Assuming a maximum error of 5 % and confidence interval 95 %, at least 117 individuals were required to the sub-sample representative of the population in question. However, in this analysis, the minimum was extrapolated to 162 individuals (an increase of approximately 40 %) to prevent sample loss during the study.

In summary, of the 829 subjects in the cross-sectional survey to screen for diabetes, 14 were excluded due to missing data, leaving 815 subjects (36.3 % men and 63.3 % women, 55.5±8.1 years and 26.9±3.7 BMI) for analysis. Among subjects with complete data, 300 subjects were randomly selected and received by post a letter of invitation to attend the Human Performance Laboratory at the University for blood collection in order to compose the sub-sample validation of a screening tool. Of these 300 subjects invited, 162 participants attended (55.5 % men and 45.5 % women, 56.9±8 years and 26.6±4 BMI) (Table 1). This was the final sample for the validation study.

All subjects in this study signed an informed consent. The study was approved by the Ethics Committee for human studies at the Viçosa Federal University.

Biochemical analysis

Blood collection was performed by a trained biochemist, using the vacuum collection technique. Blood samples were stored into the freezer and then taken for analysis. Blood samples analysis to identify A1C concentration were performed on the same day of collection at the Laboratory of Clinical Analysis, Division of Health, Viçosa Federal University, using the HPLC (High Performance Liquid Chromatography) method by the apparatus VARIANT II System (Bio-Rad Laboratories, Inc., USA).

Finnish Diabetes Risk Score (FINDRISC)

Details about the development and validation in a prospective setting and cross-sectional evaluation of the FINDRISC in Finnish population have been published elsewhere [13, 18].

The FINDRISC was produced to be a simple risk calculator that could be conveniently used in primary care and also by individuals themselves, only those variables that were easy to assess without any laboratory tests or those clinical measurements that did not require special skills were included [13].

The final risk score form is a one-page questionnaire containing eight questions, with categorized answers, about age, BMI, waist circumference, physical activity, daily

Table 1 Sample characteristics and results of A1C, estimated average glucose and FINDRISC Score

Variables	Female	Male	Total sample
n	72	90	162
Age (years)	56.5±9.0	57.3±7.2	56.9±8
Weight (kg)	66.2±10.4	74.8±12.1*	71±12.2
Height (m)	1.5±0.05	1.6±0.06*	1.6±0.08
BMI (kg/m^2)	26.8±4.6	26.4±3.5	26.6±4
WC (cm)	92±12.1	94.6±10.8	93.4±11.5
A1C (%)	5.7±0.3	5.7±0.5	5.7±0.4
eAG (mmol/l)	6.6±0.6	6.6±0.8	6.6±0.7
FINDRISC Score	11.3±4.8	11±5.4	11.1±5.1

Data are means ± SD. *Significant difference between gender (P≤ 0.001)

eAG estimated Average Glucose; A1C glycosylated hemoglobin; WC Waist Circumference

consumption of fruits, berries or vegetables, history of antihypertensive drug treatment, history of high blood glucose, and family history of diabetes. These variables predicted diabetes incidence in the original study cohort from which the risk score was developed. Each of the answers to the questions in the form was weighted, corresponding to the risk increase associated to the respective variable in the original model. The total risk score is a simple sum of the individual weight, and values range from 0 to 26 [13].

The FINDRISC was chosen as a tool to be validated in Brazil because it is currently the best screening tool available for use in clinical practice and valid in several countries [8]. Importantly, several authorities such as the European Association for the Study of Diabetes, the European Society of Cardiology, and the International Diabetes Federation Consensus Group have recommended the FINDRISC to be used for the risk stratification purposes in the European population [19, 20].

Statistical methods

The equation proposed by Lwanga and Lemeshow [15] was used for calculating the minimum sample in order to extract a sub-sample for the study of the questionnaire. The comparison of results between men and women was performed through the Mann–Whitney test using the software SigmaPlot for Windows Version 11.0 (Systat Software, Inc., Chicago, IL, USA).

The predictive performance of the risk score was evaluated with respect to the Area Under the Curve (AUC) in a Receiver Operating Characteristics (ROC) curve, sensitivity (the probability of a positive test given to the individual that truly does have the disease), specificity (the probability of a negative test given to the individual that does not have the disease), the Positive Predictive Value—PPV (the probability of the disease giving a positive test), and the Negative Predictive Value—NPV (the probability of a non-diseased giving a negative test).

We realize the ROC curve analysis of all individuals with A1C values ≥ 6.0 and the other only in patients with diabetes (A1C ≥ 6.5 %)) to determine the prevalence of individuals with IGT and diabetes.

These tests were performed using the software MedCalc® Version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium). All tests were calculated using a Confidence Interval (CI) of 95 % and $P \leq 0.05$ was assumed to indicate significance.

Definitions

As indicated by American Diabetes Association [7, 16], we used the A1C test as gold standard for the diagnosis of type 2 diabetes. The A1C test was chosen because it presents some advantages over Oral Glucose Tolerance Test (OGTT) and Fasting Plasma Glucose (FPG) for example: 1) Better index of overall glycemic exposure and risk for long-term complications; 2) Substantially less biologic variability; 3) Substantially less pre-analytic instability 4) Relatively unaffected by acute (e.g. stress or illness related) perturbations in glucose levels [16].

Individuals who had A1C ≤ 5.9 % were considered as "normal", 6.0 % to 6.4 % as "IGT", and A1C ≥ 6.5 % as "with diabetes" [16]. Studies show that the risk of diabetes increase with the increase of A1C and cutoff point ≥ 6.0 % was associated with a highly increased risk of incident diabetes [17]. Therefore, we considered the cutoff point of A1C ≥ 6.0 % as diagnostic of IGT.

Results

When analyzing the data, we divided the sub-sample by gender to ascertain if there were differences in the variables analyzed between men and women. The only existing statistical differences in variables were body weight and height, which showed higher values in men than in women (Table 1). However, these differences did not affect BMI and other variables, which showed no statistical differences (Table 1). Therefore, we consider our sub-sample as being homogeneous with respect to the variables analyzed, and all results presented below are for the total sample.

The prevalence of IGT and diabetes according to the A1C test were 21.6 % and 8.6 % respectively. The Diabetes Risk Score value varied from 1 to 25 and the area under the ROC curve (AUC) by considering all values of A1C ≥ 6.0 % was 0.69 (95 % CI 0.61–0.76) (Fig. 1). The sensitivity, specificity, PPV and NPV by each score in a sample Brazilians are showed in Table 2. Based on the best cutoff point found in this study, we could determine the prevalence of IGT in Brazilians (Table 3).

Discussion

The result of the performance of FINDRISC to identify Brazilians with IGT is satisfactory, indicating that it can be used as a screening tool. The best cutoff point found in Brazilians demonstrates a sensitivity of 75.51 % in identifying people likely to develop diabetes.

Another data that strengthens the use of FINDRISC in the prevention of diabetes is the NPV of 82.4 %, meaning that of the people who submit scores below 9, 82.4 % are not having IGT. Analyzing the data in patients with diabetes (A1C ≥ 6.5 %), resulted in a sensitivity 78.57 % (95 % CI 49.2–95.3 %), specificity of 43.92 % (95 % CI 35.8–

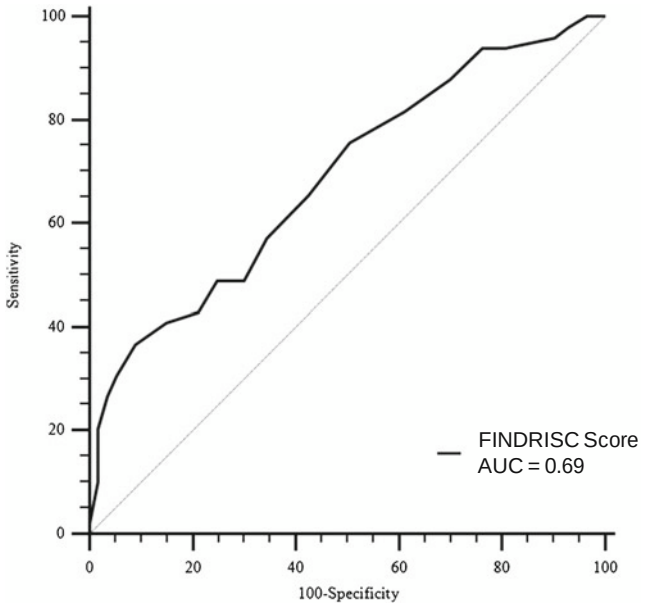

Fig. 1 ROC curves showing the performance of FINDRISC Score for identifying Brazilians with IGT (A1C≥6.0 %)

Table 2 The sensitivity, specificity, PPV and NPV by each score in Brazilians with IGT (A1C≥6.0 %)

Total score	Sensitivity	Specificity	PPV	NPV
1	100.00	0	30.2	100.0
2	100.00	3.54	31.0	100.0
3	97.96	7.08	31.4	88.9
4	95.92	9.73	31.5	84.6
5	93.88	19.47	33.6	88.0
6	93.88	23.89	34.8	90.0
7	87.76	30.09	35.2	85.0
8	81.63	38.94	36.7	83.0
9	75.51	49.56	39.4	82.4
10	65.31	57.52	40.0	79.3
11	57.14	65.49	41.8	77.9
12	48.98	69.91	41.4	76.0
13	48.98	75.22	46.2	77.3
14	42.86	78.76	46.7	76.1
15	40.82	84.96	54.1	76.8
16	36.73	91.15	64.3	76.9
17	30.61	94.69	71.4	75.9
18	26.53	96.46	76.5	75.2
19	20.41	98.23	83.3	74.0
20	10.20	98.23	71.4	71.6
21	6.12	99.12	75.0	70.9
23	2.04	100.00	100.0	70.2
25	0	100.00	100.0	69.8

Data are showed in percentages. AUC 0 0.69 (IC 95 % 0 0.61–0.76) (P ≤ 0.001). Values in bold represent best cutoff point for identifying individuals with IGT

PPV positive predictive value, NPV negative predictive value

Table 3 Prevalence of risk for identifying Brazilians with IGT (A1C≥6.0 %)

Score	Risk classification	% of study sample[a]	% of population[b]
<9	Low	32.8	30.9
≥9	High	67.2	69.1

[a] Study sample from cross-sectional survey

[b] Total population of cross-sectional survey

52.3 %), PPV of 11.7 % (95 % CI 6.0–20 %) and NPV of 95.6 % (95 % CI 87.6–99.1 %).

The present study is the first to validate a screening tool for identify people with risk to IGT in a Brazilian population. We conducted a search in two international databases (Pubmed and Science Direct) until September 2010, and noticed the absence of any study using this type of tool in our population.

Our results are reinforced by recent studies [5, 21] that used questionnaires to identify people with IGT. Schwarz et al. [5] evaluated the usefulness of the FINDRISC to predict insulin resistance in a population with an increasing diabetes risk in a cross-sectional survey (1996) and a cohort study (1997–2000). Although we know that the disease prevalence is different between populations and that this fact influenced the results of the performance analysis of the questionnaires, Schwarz et al. [5] found in the cross-sectional survey (1996) a cutoff point similar to the one found in our study. In the same cutoff point, the results of sensitivity and NPV were quite similar; however, the AUC was different, ours being somewhat lower.

These results provide further evidence for the use of FINDRISC as a tool for screening to identify Brazilian individuals with IGT. The most important use of FINDRISC is the primary health care, where strategies for population-based screening are widely needed. The use by primary care physicians or other health care professionals would facilitate the detection of high-risk subjects and the implementation of early preventive measures.

Current screening tools for type 2 diabetes include questionnaires that assess risk factors, biochemical tests or a combination of both [8]. However, many of these tools require some training prior to their use and invasive biochemical tests, mostly expensive and time consuming. Thus, the need for a simple screening tool based on risk factors for type 2 diabetes, which is self-administered, assumes paramount importance, especially in the community-based settings of developing countries.

It is well known that people with IGT have a high probability to progress into diabetes, approximately half of them will have diabetes within 10 years [22]. The rate of progression to diabetes among people with IGT depends on

their profiles of risk factors, and thus the FINDRISC can be a useful tool to identify those with IGT, which are at greater risks and which would benefit from preventive interventions, as showed recently by the FINDRISC in the Finish Diabetes Prevention Study [23].

The results of this study showed no significance between gender differences (Table 1). Considering these variables as risk factors for diabetes development, the prevalence of gender equality regarding diabetes in Brazil is noteworthy, as has been observed in previous studies [24].

The prevalence of IGT and diabetes according to the A1C test was respectively 21.6 % and 8.6 %. The prevalence of diabetes observed in this study is slightly higher than the estimated prevalence (6.4 %) by International Diabetes Federation (IDF) [2]. However, recent studies done by the Ministry of Health [1] shows that the prevalence of diabetes in Brazil increased from 5.2 % in 2006 to 5.8 % in 2009.

In a recently published study on data from the CARMELA Study [25] was found that the prevalence of diabetes and impaired fasting glucose is high in the seven largest cities in Latin America. Overall, the prevalence of diabetes was 7.0 % (95 % CI 6.5–7.6) and impaired fasting glucose was found in 2 % of the population. However, the data related to the Brazilian population were not included in this study.

When we compared our prevalence data with the CARMELA Study [25], we found that our results are quite close to those found in Mexico City (8.9 % CI 7.7 to 10.2) and Bogota (8.1 %; 95 % CI 6.8 to 9.5). In relation to IGT condition, results of Viçosa city are ten times the ones found in the CARMELA Study [25]. This demonstrates the importance of early diagnosis of people at risk for developing type 2 diabetes, because changes in the lifestyle can prevent or delay the onset of type 2 diabetes in the population [22].

The strength of our study lies in the fact that randomization of the study sample was done and a standard and specific test was used for the diagnosis. The main limitation of our study is the small sample size used to validate the questionnaire. However, the randomization of the study sample potentially decreased the chances of errors. Our sample size calculation was performed by the equation proposed by Lwanga and Lemeshow [15], which is recommended by the World Health Organization (WHO) for epidemiological studies.

In conclusion, our analysis shows that the FINDRISC demonstrated to be a suitable tool to identify Brazilians with IGT.

When implemented in primary health care, the FINDRISC would help health care professionals in decision making regarding the need for further medical investigation and the institution of preventive measures. Furthermore, the application of the FINDRISC in a population-based program also produces a learning effect. People completing the FINDRISC become aware of their own prevalent risk factor.

As proposed by several authorities such as the European Association for the Study of Diabetes, the European Society of Cardiology, and the International Diabetes Federation Consensus Group, the FINDRISC is a useful tool to be used for risk stratification purposes [19, 20]. Brazil, as a developing country of continental dimension, also has a poor health care system, in which a certain percentage of the population lacks access to appropriate health care services and therefore could benefit from using the FINDRISC in primary health care.

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

References

1. Vigitel Brazil 2009: protection and risk factors for chronic diseases by telephone inquiry. 2010. http://portal.saude.gov.br/portal/arquivos/pdf/vigitel2009_220610.pdf. Accessed 2 Oct 2010.
2. International Diabetes Federation - Prevalence estimates of diabetes mellitus (DM), 2010. 2010. http://www.diabetesatlas.org/sites/default/files/DM%202010_7%20regions.xls. Accessed 02 Nov 2010.
3. International Diabetes Federation - Economic impact of Diabetes. 2010. http://www.diabetesatlas.org/sites/default/files/ Economic%20impact%20of%20Diabetes.pdf. Accessed 2 Nov 2010.
4. International Diabetes Federation - Prevalence estimates of diabetes mellitus (DM), 2030. 2010. http://www.diabetesatlas.org/sites/default/files/DM%202030_7%20regions.xls. Accessed 02 Nov 2010.
5. Schwarz PE, Li J, Reimann M, Schutte AE, Bergmann A, Hanefeld M, Bornstein SR, Schulze J, Tuomilehto J, Lindstrom J. The Finnish Diabetes Risk Score is associated with insulin resistance and progression towards type 2 diabetes. J Clin Endocrinol Metab. 2009;94:920–6.
6. Screening for type 2 diabetes. Diabetes Care. 2004;27 Suppl 1:S11–4.
7. Standards of medical care in diabetes—2010. Diabetes Care. 2010; 33 Suppl 1:S11–61.
8. Schwarz PE, Li J, Lindstrom J, Tuomilehto J. Tools for predicting the risk of type 2 diabetes in daily practice. Horm Metab Res. 2008;41:86–97.
9. Gao WG, Qiao Q, Pitkaniemi J, Wild S, Magliano D, Shaw J, Soderberg S, Zimmet P, Chitson P, Knowlessur S, et al. Risk prediction models for the development of diabetes in Mauritian Indians. Diabet Med. 2009;26:996–1002.
10. Gao WG, Dong YH, Pang ZC, Nan HR, Wang SJ, Ren J, Zhang L, Tuomilehto J, Qiao Q. A simple Chinese risk score for undiag- nosed diabetes. Diabet Med. 2010;27:274–81.
11. Cabrera de Leon A, Coello SD, Rodriguez Perez Mdel C, Medina MB, Almeida Gonzalez D, Diaz BB, de Fuentes MM, Aguirre- Jaime A. A simple clinical score for type 2 diabetes mellitus screen- ing in the Canary Islands. Diabetes Res Clin Pract. 2008;80:128–33.

12. Glumer C, Vistisen D, Borch-Johnsen K, Colagiuri S. Risk scores for type 2 diabetes can be applied in some populations but not all. Diabetes Care. 2006;29:410–4.
13. Lindstrom J, Tuomilehto J. The diabetes risk score: a practical tool to predict type 2 diabetes risk. Diabetes Care. 2003;26:725–31.
14. Brazilian Institute of Geography and Statistics. Population census summary. http://www.ibge.gov.br/cidadesat/topwindow.htm. Accessed 2 Apr 2011.
15. Lwanga SK, Lemeshow S. Sample size determination in health studies: a practical manual. Geneva: World Health Organization; 1991.
16. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. Diabetes Care. 2009;32:1327–34.
17. Zhang X, Gregg EW, Williamson DF, Barker LE, Thomas W, Bullard KM, Imperatore G, Williams DE, Albright AL. A1C level and future risk of diabetes: a systematic review. Diabetes Care. 2010;33:1665–73.
18. Saaristo T, Peltonen M, Lindstrom J, Saarikoski L, Sundvall J, Eriksson JG, Tuomilehto J. Cross-sectional evaluation of the Finnish Diabetes Risk Score: a tool to identify undetected type 2 diabetes, abnormal glucose tolerance and metabolic syndrome. Diab Vasc Dis Res. 2005;2:67–72.
19. Schwarz PE, Lindstrom J, Kissimova-Scarbeck K, Szybinski Z, Barengo NC, Peltonen M, Tuomilehto J. The European perspective of type 2 diabetes prevention: diabetes in Europe–prevention using lifestyle, physical activity and nutritional intervention (DE-PLAN) project. Exp Clin Endocrinol Diabetes. 2008;116:167–72.
20. Schwarz PE, Gruhl U, Bornstein SR, Landgraf R, Hall M, Tuomilehto J. The European perspective on diabetes prevention: development and Implementation of A European Guideline and training standards for diabetes prevention (IMAGE). Diab Vasc Dis Res. 2007;4:353–7.
21. Tuomilehto J, Lindstrom J, Hellmich M, Lehmacher W, Westermeier T, Evers T, Bruckner A, Peltonen M, Qiao Q, Chiasson JL. Development and validation of a risk-score model for subjects with impaired glucose tolerance for the assessment of the risk of type 2 diabetes mellitus-The STOP-NIDDM risk-score. Diabetes Res Clin Pract. 2010;87:267–74.
22. Lindstrom J, Ilanne-Parikka P, Peltonen M, Aunola S, Eriksson JG, Hemio K, Hamalainen H, Harkonen P, Keinanen-Kiukaanniemi S, Laakso M, et al. Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study. Lancet. 2006;368:1673–9.
23. Lindstrom J, Peltonen M, Eriksson JG, Aunola S, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukaanniemi S, Uusitupa M, Tuomilehto J. Determinants for the effectiveness of lifestyle intervention in the Finnish Diabetes Prevention Study. Diabetes Care. 2008;31:857–62.
24. Goldenberg P, Schenkman S, Franco LJ. Prevalence of diabetes mellitus: gender differences and sex equalities. Rev Bras Epide- miol. 2003;6:18–28.
25. Escobedo J, Buitron LV, Velasco MF, Ramirez JC, Hernandez R, Macchia A, Pellegrini F, Schargrodsky H, Boissonnet C, Champagne BM. High prevalence of diabetes and impaired fasting glucose in urban Latin America: the CARMELA Study. Diabet Med. 2009;26:864–71.

ORIGINAL ARTICLE

Inflammatory markers in metabolic syndrome

M. K. Garg & M. K. Dutta & K. S. Brar

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Abstract The Metabolic Syndrome (MS) is associated with a systemic inflammatory response that plays an important pathogenetic role in atherothrombotic disease. Highly sensitive C-reactive protein (hsCRP) and fibrinogen are acute phase reactants and indicate underlying inflammatory state. We studied inflammatory markers in 50 Indian subjects with MS diagnosed by IDF criteria and 24 healthy age and sex matched controls. Clinical evaluation included anthropometry, body fat analysis by bio-impedance, biochemical, hsCRP, fibrinogen and insulin measurement. Subjects with MS had higher hsCRP (4.07±1.72 vs. 2.09±0.98 mg/L, P00.0006); and fibrinogen levels (336±77 vs. 193±43 mg/dl, P <0.001) than controls. hsCRP and fibrinogen levels increased with number of metabolic abnormalities. Both inflammatory markers were positively associated with body mass index, body fat mass, percent body fat, HOMA-IR and all parameters of MS, except HDL with which only fibrinogen was negatively associated. Waist hip ratio was an independent predictor of hsCRP and fibrinogen in multiple regression analysis. hsCRP level of 2.6 mg/L predicted the MS with sensitivity, specificity and accuracy of 71 %, 78 % and 75 % respectively. Subjects with MS have increased inflammatory markers compared to healthy controls.

Keywords Metabolic Syndrome · Highly sensitive C-reactive protein · Fibrinogen

M. K. Garg (*)
mkgargs@gmail.com

Introduction

Increased oxidative and inflammatory stress are recognized as playing an important role in the initiation and progression of atherosclerotic vascular disease [1, 2]. Obesity is associated with increased circulating markers of oxidative stress and low-grade inflammation [3]. The MS which often accompanies obesity, has also been independently linked with increased oxidative stress and inflammatory burden [4]. MS is associated with a pro-inflammatory state in which the role of visceral obesity is thought to be central. Inflammatory cytokines, in particular interleukin (IL)-1, tumor necrosis factor, and IL-6, are the main inducers of the acute phase response. Two important acute-phase proteins, highly sensitive C-reactive protein (hsCRP) and fibrinogen have IL-6 response elements in the promoter regions of their genes [5, 6]. Numerous studies have now confirmed that CRP levels are elevated in subjects with MS [7–11]. CRP levels were shown to be strongly associated with insulin resistance calculated from the HOMA model, blood pressure, low HDL, and triglycerides [10]. There is a linear relationship between the number of metabolic features and increasing levels of hsCRP [12–15]. The strongest associations were observed between CRP levels, central adiposity, and insulin resistance [16, 17]. Collectively, all of these studies support the hypothesis that an increased hsCRP in the setting of MS confers an increased risk of future CV events [7, 8, 18]. Hence, we studied inflammatory markers (hsCRP and fibrinogen) to confirm that those established associations are also present within the population of Indian subjects with MS.

Material and methods

This study was carried out at the department of Endocrinology in a tertiary care centre. Subjects between age 030 years

and ≤50 years (to exclude all postmenopausal women) were screened for the presence of MS according to IDF criteria [19] as follows:- Central obesity (Waist circumference – Male >90 cm, Female >80 cm) plus any two: raised triglycerides (>150 mg/dL), reduced HDL-cholesterol (<40 mg/dL in men or <50 mg/dL in women), raised blood pressure (Systolic ≥130 mm Hg or Diastolic ≥85 mm Hg), raised fasting plasma glucose (Fasting plasma glucose ≥100 mg/dL). Age and sex matched healthy subjects were screened for absence of metabolic syndrome. Only those cases with normal waist circumference and absence of at least three of four parameters were included.

A total of 50 drug naïve subjects with metabolic syndrome (25 males and 25 females) and 24 controls (12 males and 12 females) were included in the study. All underwent clinical examination. Subjects with hepatic disease, renal disease, other endocrine diseases, alcoholism, infectious diseases or those receiving any medications were excluded from the study.

Body Mass Index (BMI) was calculated by weight in kilogram divided by square of height in meters. Fasting blood samples were drawn for the estimation of fasting plasma glucose, renal and hepatic parameters, glycated hemoglobin (A1C), lipid profile, hsCRP and fibrinogen. One aliquot was frozen at −80 °C for measurement of plasma insulin. Urine spot samples were collected for measurement of urine microalbumin. The study was approved by the ethics committee of Army Hospital (Research & Referral), Delhi cantt, and all subjects gave written informed consent.

Body fat measurement was done using InBODY composition analyser-biospacer manufactured by M/S Biodex Medical Systems Inc, New York. It measured waist hip ratio (WHR), body fat mass (BFM), percent body fat (PBF) and basal metabolic rate (BMR). Biochemical estimations were carried out using automated analyzer (Beckman Coulter, Synchrone CX-9 PRO, fully automated biochemistry analyzer, USA) and commercial kits (DiaSys Diagnostic systems, Germany). The normal range for different biochemical parameters are as follows: fasting plasma glucose (70–100 mg/dL), serum creatinine (0.6–1.6 mg/dL), total cholesterol (TC, <240 mg/dl), serum triglycerides (TG, <150 mg/dl), HDL-cholesterol (HDL, >40 mg/dl for males and >50 mg/dl for females), and LDL-cholesterol (calculated) (LDL, <160 mg/dl), fibrinogen (150–400 mg/dl). A1C was measured by HPLC method using commercial kit ClinRep®, Recipe Chemicals and Instruments, Germany, which was calibrated to value level of DCCT. Intra-assay and inter-assay precision was 1–2 % and 3 % respectively. Plasma insulin levels were measured by immune-radiometric-assay using Immunotech, Czech Republic, commercial kits, with measurement range 0.5–300 μIU/ml and normal value 2.1–22 μIU/ml. It had sensitivity of 0.5μIU/ml. Intra-assay and inter-assay coefficient of variation were 4.3 % and 3.4 % respectively. The HOMA model was used to calculate insulin resistance (HOMA-IR). hsCRP levels were measured by commercial kit CardioPhase® Seimens Healthcare System, USA, based on particle enhanced immunonephelometry. Sensitivity was 0.175 mg/L, with coefficient of variation 7.6 %.

Statistical analysis was carried out using EPI INFO 3.5.3 (CDC, Atlanta, GA, USA). Data were presented as mean ± SD or number (%) unless specified. All parametric data were analyzed by student'st-test. If Barlett's chi-square test for equality of population variances was <0.05 then Kruskal-Wallis test applies. All non-parametric data were analyzed by chi-square test. Multiple regression analysis was done to ascertain association between various parameters. A P value of <0.05 was considered statistically significant.

Results

This study was carried out in 50 cases of metabolic syndrome and 24 normal healthy controls. Basal characteristics of cases and controls are depicted in Table 1. BMI¸ Body fat mass and percent body fat were significantly higher in cases than controls. However, cases had significantly lower basal metabolic rate than controls. There were 34 (68 %) cases with T2DM and 14 (32 %) cases with Impaired Glucose Tolerance (IGT). All controls had normal glucose tolerance. Hypertension was present in 26 cases (52 %) among cases and none among controls. Among cases TG¸ TC¸ and LDL were significantly higher and HDL was significantly lower than controls. However¸ 14 controls (58 %) also had low HDL. Most of the cases (28, 56 %) had four features of MS followed by all features (16, 32 %) and 6 (12 %) cases had three features (Table 1).

C-reactive protein

Subjects with MS had significantly higher hsCRP levels than controls (4.07±1.72 vs. 2.09±0.98 mg/L, P00.0006). hsCRP levels increased with increasing number of metabolic abnormalities (Fig. 1). There was no difference in hsCRP levels between sexes (3.17±1.85 vs. 3.13±1.67 mg/L, P00.15).

In univariate regression analysis hsCRP was positively associated with BMI, body fat mass and percent body fat, and negatively with basal metabolic rate. Among various parameters of MS, hsCRP was positively associated with all parameters of MS, except HDL. hsCRP showed strong positive association with fibrinogen, HOMA-IR (Table 2).

During multiple regression analysis among metabolic parameters, hsCRP maintained positive association with WHR and triglycerides even after adjustment for all factors. However HDL, FPG and hypertension lost significance during multiple regression analysis (Table 3). Only percent body fat remained positively associated with hsCRP when adjusted for BMR, however, lost significance when BMI and BFM was added in multiple regression analysis (Table 4).

Table 1 Basic Characteristics of Cases and Controls

Parameters	Cases (N0 50)	Controls (N0 24)	P-value
Age	43.4±5.3	41.9±4.0	0.21
WHR			
Male	1.18±0.1	0.84±0.04	<0.0001
Female	1.21±0.11	0.74±0.03	<0.0001
BMI	28.1±2.1	22.5±2.3	<0.0001
Body fat mass	28.9±11.5	12.9±4.1	<0.0001
Body Fat (%)	34.3±7.0	18.4±4.3	<0.0001
Basal Metabolic Rate	1435±134	1740±119	<0.0001
Hypertension	26 (52 %)*	-	
Glycemic Status			
Fasting PG (mg/dl)	136±37	87±6	<0.0001
Post-glucose PG (mg/dl)	207±51	111±22	<0.0001
A1C (%)	7.9±0.9	5.0±0.3	<0.0001
Insulin (μIU/ml)	10.7±10.2	8.2±2.38	
Insulin (Median)	10.24	7.30	0.44
DM/IGT (%)	34 (68 %)/16 (32 %)*	-	
Lipid Profile			
Triglycerides (mg/dl)	200±67 (47,94 %)*	92±30	<0.0001
HDL (mg/dl)	36±6 (42,84 %)*	45±13 (14,58 %)*	<0.0001
Total Cholesterol (mg/dl)	221±40	161±33	<0.0001
LDL (mg/dl)	145±47	109±19	<0.0001
VLDL (mg/dl)	40±14	20±17	<0.0001
Urine Microalbumin	6.94±1.62	6.86±1.65	0.85
Metabolic Features			
None	-	10 (42 %)*	
One	-	13 (58 %)*	
Three	6 (12 %)*	-	
Four	28 (56 %)*	-	
Five	16 (32 %)*	-	

WHR waist hip ratio; BMI body mass index; PG plasma glucose; A1C glycated hemoglobin A1C; DM diabetes mellitus; IGT impaired glucose tolerance; HDL high density lipoprotein; LDL low density lipoprotrin; VLDL very low density lipoprotein
* number (%)

Receiver Operating Characteristic (ROC) curve analysis revealed best prediction value of hsCRP at 2.6 mg/L with sensitivity of 71 %, specificity of 78 %, accuracy of 75 %, positive predictive value of 60 %, negative predictive value of 78 % and positive and negative likelihood ratio of 3.21 and 0.37 respectively. (ROC 0.835, SE 0.0489, 95 % CI −0.739–0.931) (Fig. 2). If cut off point of hsCRP was taken as 3.0 mg/L sensitivity increased to 75 %, but specificity decreased to 72 % with accuracy of 72 % and positive predictive value of 56 % and negative predictive value of 72 %.

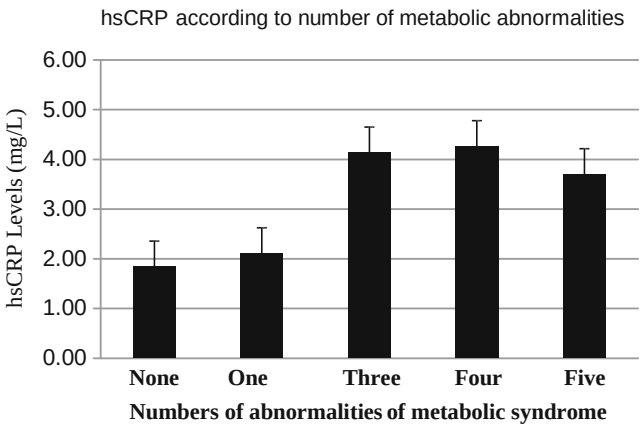

Fig. 1 hsCRP according to number of metabolic abnormalities

Fibrinogen

Subjects with MS had significantly higher fibrinogen levels than controls (336±77 vs. 193±43 mg/dl, P <0.001). Fibrinogen levels increased with increasing number of metabolic abnormalities (Fig. 3). There was no difference in fibrinogen levels between sexes (301±93 vs. 278±98 mg/dl, P0.31).

In univariate regression analysis fibrinogen was positively associated with BMI, body fat mass and percent body fat, and negatively with basal metabolic rate. Among various parameters of MS, fibrinogen was positively associated with all parameters of MS, except HDL with which it was negatively

Table 2 Univariate Regression analysis of inflammatory markers among all subjects

Parameters	hsCRP		Fibrinogen	
	Beta Coefficient (r^2 value)	P-value	Beta Coefficient (r^2 value)	P-value
Age	−0.012 (0.0)	0.786	0.033 (0.03)	0.141
Sex	−0.381 (0.0)	0.712	0.227 (0.01)	0.31
BMI	0.20 (0.30)	<0.00001	0.086 (0.20)	0.00007
Body fat mass	0.082 (0.32)	<0.00001	0.032 (0.17)	0.0003
Percent body fat	0.112 (0.38)	<0.00001	0.045 (0.21)	0.00004
Basal metabolic rate	−0.004 (0.24)	<0.00001	−0.002 (0.20)	0.00007
Metabolic Syndrome				
WHR	16.265 (0.49)	<0.00001	3.034 (0.36)	<0.00001
Fasting PG	0.055 (0.23)	0.00002	0.013 (0.26)	<0.00001
Hypertension	3.578 (0.15)	0.0007	0.986 (0.24)	0.00001
Triglycerides	0.010 (0.18)	0.0001	0.006 (0.20)	0.00005
HDL	−0.040 (0.05)	0.066	−0.038 (0.14)	0.0009
Other Parameters				
Total Cholesterol	0.008 (0.04)	0.081	0.009 (0.20)	0.00007
LDL	0.002 (0.0)	0.680	0.006 (0.08)	0.018
HOMA-IR	0.223 (0.11)	0.003	0.127 (0.13)	0.001
Fibrinogen	0.739 (0.16)	0.004	0.214 (0.16)	0.0004

BMI Body mass index; WHR waist hip ratio; PG plasma glucose; HDL high density lipoprotein; LDL low density lipoprotein; HOMA-IR homeostatic model analysis - insulin resistance

associated. Fibrinogen level showed strong positive association with total cholesterol, LDL cholesterol, hsCRP, and HOMA-IR (Table 2).

During multiple regression analysis among metabolic parameters, fibrinogen maintained positive association with WHR and FBG even after adjustment for all factors. However HDL, triglyceride and hypertension lost significance during multiple regression analysis (Table 5). Percent body fat lost significance when BMR was added. However, BMR maintained negative association with fibrinogen when adjusted for other anthropometric parameters in multiple regression analysis (Table 6).

Table 3 Multivariate Regression analysis of hsCRP with metabolic parameters among all subjects

Parameters	Beta coefficient	P-value
WHR	16.265	<0.00001
WHR + HDL	4.553	0.00002
WHR + HDL + Triglyceride	3.625	0.001
WHR + HDL + Triglyceride + FPG	4.584	0.0004
WHR + HDL + Triglyceride + FPG	0.006	0.036
WHR + HDL + Triglyceride + FPG + Hypertension	4.501	0.0004
WHR + HDL + Triglyceride + FPG + Hypertension	0.006	0.035

* Beta Coefficient and P-value for parameters in Bold

WHR waist hip ratio; HDL high density lipoprotein; FPG fasting plasma glucose

Discussion

MS is known to strongly predict long-term risk of diabetes and CVD and have also been reported to experience increased morbidity and mortality [20]. It is becoming increasingly common in the United States and worldwide and is emerging as the dominant risk factor in Asia [21]. There is high prevalence of MS (~18–26 %) in different parts of India according to different criteria [22]. Although multiple influences contribute to MS, the syndrome appears to be relatively uncommon in the absence of some excess body fat. As obesity increases so does the prevalence of MS. Excess adipose tissue releases a variety of factors including IL-6, which increases acute phase reactants like hsCRP and fibrinogen, which is linked to atherosclerosis [5].

In this study we evaluated 50 subjects with metabolic syndrome (25 males and 25 females) and 24 controls (12

Table 4 Multivariate Regression analysis of hsCRP with Anthropometric Parameters among all subjects

Parameters	Beta coefficient	P-value
Percent Body Fat	0.112	<0.00001
Percent Body Fat + BMR	0.099	0.0001
Percent Body Fat + BMR + BMI	0.090	0.055
Percent Body Fat + BMR + BMI + BFM	0.060	0.27

* Beta Coefficient and P-value for parameters in Bold

BMR basal metabolic rate; BMI body mass index; BFM body fat mass

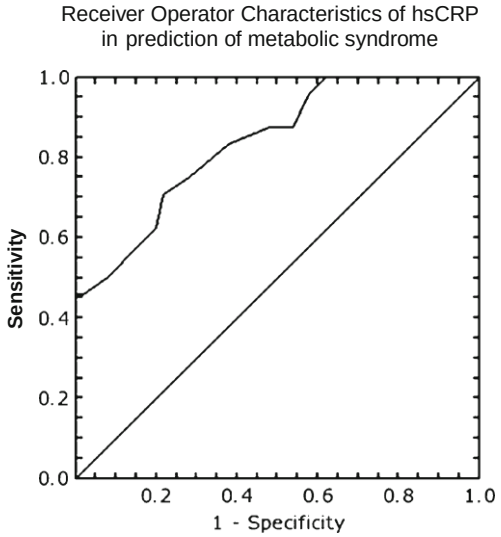

Fig. 2 Receiver Operator Characteristics of hsCRP in prediction of metabolic syndrome

males and 12 females). Different definitions have been proposed for MS [20], we have used IDF criteria as it provides ethnic specific criteria for central obesity. All cases had significantly higher WHR, BMI¸ body fat mass and percent body fat than controls in both sexes, which is similar to Asian Indian obesity phenotype [23]. However, cases had significantly lower basal metabolic rate than controls. Contrary to this, one study reported higher BMR in morbidly obese subjects with metabolic syndrome [24].

Subjects with MS had significantly higher level of hsCRP and fibrinogen than controls. This supports the hypothesis that MS is a pro-inflammatory state [24, 25]. Higher levels of hsCRP and fibrinogen in MS compared to controls have been reported in most of the studies in the literature [2, 4, 7, 10–15]. There was no difference in hsCRP level between sexes in this study, but one study reported higher hsCRP level in women [26]. Fibrinogen

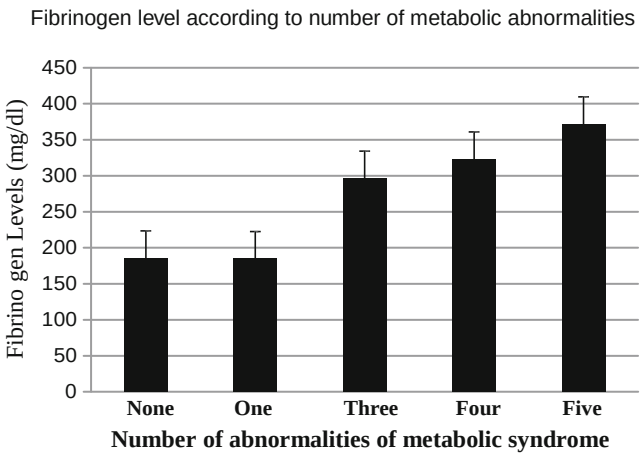

Fig. 3 Fibrinogen level according to number of metabolic abnormalities

Table 5 Multivariate Regression analysis of fibrinogen with metabolic parameters among all subjects

Parameters	Beta coefficient	P-value
WHR	3.034	<0.00001
WHR +HDL	2.70	<0.00001
WHR+HDL	−0.02	0.039
WHR +HDL+Triglyceride	2.319	0.00005
WHR +HDL+Triglyceride+FPG	1.87	0.001
WHR+HDL+Triglyceride+FPG	0.006	0.017
WHR +HDL+Triglyceride+FPG+ Hypertension	1.598	0.008

* Beta Coefficient and P-value for parameters in Bold

WHR waist hip ratio; HDL high density lipoprotein; FPG fasting plasma glucose

and hsCRP levels increased with increasing number of metabolic abnormalities. Similar relation has been eported in various studies [5, 11–15, 27]. In univariate regression analysis both markers were positively associated with BMI, body fat mass and percent body fat, cholesterol, LDL, HOMA-IR and negatively with basal metabolic rate. Vikram et al. [28] made similar observation about relation between hsCRP and BMI, and WHR; but found no relation between hsCRP and HOMA-IR and lipid parameters in Indian young teenagers. Another study [12] among South Indian subjects with metabolic syndrome with normal glucose tolerance reported lower hsCRP level than present study (2.7±2 vs.14.07±1.72); which can be explained by the fact that hsCRP levels increases in diabetics [29]. Lower levels of hsCRP have been reported in Japanese [30] and Chinese [15] than Caucasians and Indians; however relation with metabolic syndrome persisted within population. Among various parameters of MS, both markers were positively associated with WHR and hypertension. Festa et al. [10] from the Insulin Resistance and Atherosclerosis Study showed that hsCRP was positively correlated with BMI, waist circumference, blood pressure, triglycerides, cholesterol, LDL cholesterol, plasma glucose, and fasting insulin and inversely correlated with HDL cholesterol and insulin sensitivity index.

Table 6 Multivariate Regression analysis of CRP with Anthropometric Parameters among all subjects

Parameters	Beta coefficient	P-value
Percent Body Fat	0.045	0.00007
Percent Body Fat +BMR	0.028	0.056
Percent Body Fat+BMR +BMI	−0.001	0.058
Percent Body Fat+BMR +BMI+BFM	−0.002	0.05

* Beta Coefficient and P-value for parameters in Bold

BMR basal metabolic rate; BMI body mass index; BFM body fat mass

The strongest associations were observed between CRP levels, central adiposity, and insulin resistance. The largest study to date that examined the association between inflammation and MS was the third NHANES study [11]. In a representative sample of the US population (8,570 participants >20 years of age), subjects with the MS, defined by using ATP-III criteria, were more likely than those without the syndrome to have elevated levels of markers of inflammation such as hsCRP, fibrinogen, and leukocyte count. Thus, there appears to be a clear relationship between the number of metabolic features and increasing hsCRP levels. Kraja et al. [25] also found that CRP had a significant correlation with fibrinogen (r00.46). The strongest associations were observed between CRP levels, central adiposity, and insulin resistance. Unek et al. [30] also found positive correlation of hsCRP with waist circumferences, fasting blood glucose, postprandial blood glucose, and glycated hemoglobin, and negative correlation with HDL cholesterol. During multiple regression analysis among metabolic parameters, WHR and triglycerides maintained positive association with CRP even after adjustment for all factors. However HDL, FPG and hypertension lost significance during multiple regression analysis. ROC curve analysis revealed best prediction value at 2.6 mg/L with sensitivity of 71 %, specificity of 78 %, accuracy of 75 %, and positive predictive value of 60 %. If cut off point was taken as 3.0 mg/L sensitivity increased to 75 %, but specificity decreased to 72 % with accuracy of 72 % and positive predictive value of 56 %. In the WOSCOPS, in which 6,447 men were followed for 4.9 years, an hsCRP level >3 mg/L predicted greater CVD risk in patients with MS in a multivariate model [16]. Oda et al. [29] analyzed data of hsCRP and WBC from 2,185 Japanese men and 1,383 Japanese women using ROC curve for diagnosing MS. The area under ROC curve (AUC) of hsCRP was 0.71 in men and 0.74 in women which is similar to present study. In multiple logistic regression models, hsCRP showed significant associations with MS after controlling for cardiovascular risk factors [14].

The primary limitation of the present study is its cross-sectional design and the inherent possibility that genetic and/or lifestyle factors may have influenced the results of our group comparisons. However, in an effort to minimize the influence of lifestyle behaviors, we studied subjects of similar age who were non-smokers, who were not currently taking medication that could influence hsCRP or fibrinogen levels.

In conclusion, subjects with MS have increased inflammatory markers as compared to healthy controls. Inflammatory markers are positively associated with BMI, WHR, body fat mass, percent body fat, cholesterol and various other parameters of MS. Life style measures have been shown to improve inflammatory parameters and various components and effects of metabolic syndrome [31–33], hence there is an urgent need to combat the epidemic of MS in India by public health measures to prevent ongoing epidemic of diabetes and CVD.

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Conflicts of interest There is no conflict of interest.

References

1. Hansel B, Giral P, Nobecourt E, Chantepie S, Bruckert E, Chapman MJ, et al. Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. J Clin Endocrinol Metab. 2004;89:4963–71.
2. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: A comprehensive perspective based on interactions between obesity, diabetes and inflammation. Circulation. 2005;111:1448–54.
3. Festa A, D'Agostino Jr R, Williams K, Karter AJ, Mayer-Davis EJ, Tracy RP, et al. The relation of body fat mass and distribution to markers of chronic inflammation. Int J Obes Relat Metab Disord. 2001;25:1407–15.
4. Yajnik CS, Joglekar CV, Lubree HG, Rege SS, Naik SS, Bhat DS, et al. Adiposity, inflammation and hyperglycaemia in rural and urban Indian men: Coronary Risk of Insulin Sensitivity in Indian Subjects (CRISIS) Study. Diabetologia. 2008;51:39–46.
5. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflam- mation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis. 2000;148:209–14.
6. Vozarova B, Weyer C, Hanson K, Tataranni PA, Bogardus C, Pratley RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obes Res. 2001;9:414–7.
7. Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14719 initially healthy American women. Circulation. 2003;107:391–7.
8. Ballantyne CM, Hoogeveen RC, Bang H, Coresh J, Folsom AR, Chambless LE, et al. Lipoprotein-associated phospholipase A2, high-sensitivity C-reactive protein, and risk for incident ischemic stroke in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study. Arch Intern Med. 2005;165:2479–84.
9. Sugiura K, Tamakoshi K, Yatsuya H, Otsuka R, Wada K, Matsushita K, et al. Contribution of adipocytokines to low-grade inflammatory state as expressed by circulating C-reactive protein in Japanese men: comparison of leptin and adiponectin. Int J Cardiol. 2008;130:159–64.
10. Festa A, D'Agostino Jr R, Howard G, Mykkänen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). Circulation. 2000;102:42–7.
11. Vu JD, Vu JB, Pio JR, Malik S, Franklin SS, Chen RS, et al. Impact of C-reactive protein on the likelihood of peripheral arterial disease in United States adults with the metabolic syndrome, diabetes mellitus, and preexisting cardiovascular disease. Am J Cardiol. 2005;96:655–8.
12. Gokulakrishnan K, Deepa R, Sampathkumar R, Balasubramanyam M, Mohan V. Association of leukocyte count and hsCRP with metabolic abnormalities in subjects with normal glucose tolerance (CURES - 64). J Assoc Phys India. 2009;57:27–32.

13. Huffman FG, Gomez GP, Zarini GG. Metabolic syndrome and high- sensitivity C-reactive protein in Cubans. Ethn Dis. 2009;19:115–20.
14. Ebrahimi A, Nabipour I, Vahdat K, Jafari SM, Fouladvand M, Assadi M, et al. High sensitivity C-reactive protein is associated with the metabolic syndrome independent to viral and bacterial pathogen burden. Diabetes Res Clin Pract. 2009;84:296–302.
15. Wen J, Liang Y, Wang F, Sun L, Guo Y, Duan X, et al. Association of C-reactive protein and metabolic syndrome in a rural Chinese population. Clin Biochem. 2009;42:976–83.
16. Ridker PM, Wilson PW, Grundy SM. Should C-reactive protein be added to metabolic syndrome and to assessment of global cardio- vascular risk? Circulation. 2004;109:2818–25.
17. Kressel G, Trunz B, Bub A, Hülsmann O, Wolters M, Lichtinghagen R, et al. Systemic and vascular markers of inflammation in relation to metabolic syndrome and insulin resistance in adults with elevated atherosclerosis risk. Atherosclerosis. 2009;202:263–71.
18. Hansson GK. Inflammation, atherosclerosis and coronary artery disease. N Engl J Med. 2005;352:1685–95.
19. Alberti KGMM, Zimmet P, Shaw J. IDF Epidemiology Task Force Consensus Group. Lancet. 2005;66:1059–62.
20. Lorenzo C, Williams K, Hunt KJ, Haffner SM. The National Cholesterol Education Program - Adult Treatment Panel III, International Diabetes Federation, and World Health Organiza- tion definitions of the metabolic syndrome as predictors of incident cardiovascular disease and diabetes. Diabetes Care. 2007;30:8–13.
21. Reddy KS. Cardiovascular diseases in the developing countries: Dimensions, determinants, dynamics and directions for public health action. Publ Health Nutr. 2001;5:231–7.
22. Mohan V, Deepa M. Prevalence of diabetes and metabolic syndrome among Asians (Editorial). Int J Diabetes Dev Ctries. 2010;30:173–5.
23. Misra A, Vikram NK. Insulin resistance syndrome (metabolic syndrome) and Asian Indians. Curr Sci. 2002;83:1483–96.
24. Tarantino G, Marra M, Contaldo F, Pasanisi F. Basal metabolic rate in morbidly obese patients with non-alcoholic fatty liver disease. Clin Invest Med. 2008;31:E24–9.
25. Kraja AT, Province MA, Arnett D, Wagenknecht L, Tang W, Hopkins PN, et al. Do inflammation and procoagulation bio- markers contribute to the metabolic syndrome cluster? Nutr Metab (Lond). 2007;4:28–34.
26. Saltevo J, Vanhala M, Kautiainen H, Kumpusalo E, Laakso M. Gender differences in C-reactive protein, interleukin-1 receptor antagonist and adiponectin levels in the metabolic syndrome: a population-based study. Diabet Med. 2008;25:747–50.
27. Onat A, Can G, Hergenç G. Serum C-reactive protein is an inde- pendent risk factor predicting cardiometabolic risk. Metabolism. 2008;57:207–14.
28. Vikram NK, Misra A, Pandey RM, Dwivedi M, Luthra K, Dhingra V, et al. Association between subclinical inflammation & fasting insulin in urban young adult north Indian males. Indian J Med Res. 2006;124:677–82.
29. Oda E, Kawai R. Comparison between high-sensitivity C-reactive protein (hs-CRP) and white blood cell count (WBC) as an inflam- matory component of metabolic syndrome in Japanese. Intern Med. 2010;49:117–24.
30. Unek IT, Bayraktar F, Solmaz D, Ellidokuz H, Yuksel F, Sisman AR, et al. Enhanced levels of soluble CD40 ligand and C-reactive protein in a total of 312 patients with metabolic syndrome. Metab- olism. 2010;59:305–13.
31. Singhal N, Misra A, Shah P, Gulati S, Bhatt S, Sharma S, et al. Impact of intensive school-based nutrition education and lifestyle interventions on insulin resistance, β-cell function, disposition index, and subclinical inflammation among Asian Indian adoles- cents: a controlled intervention study. Metab Syndr Relat Disord. 2011;9:143–50.
32. Esposito K, Pontillo A, Di Palo C, Giugliano G, Masella M, Marfella R, et al. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. JAMA. 2003;289:1799–804.
33. Balagopal P, George D, Patton N, Yarandi H, Roberts WL, Bayne E, et al. Lifestyle-only intervention attenuates the inflammatory state associated with obesity: a randomized controlled study in adolescents. J Pediatr. 2005;146:342–8.

ORIGINAL ARTICLE

Evaluation of anti-diabetic effect of Trigonella foenum graecum Linn. Leaf extract in streptozotocin induced diabetic rats

Ramya Premanath & N. Lakshmidevi & K. Jayashree & R. N. Suresh

Int J Diab Dev Ctries. 2012; 32: 138-144

Abstract Trigonella foenum graecum leaves are widely used as a vegetable throughout India and have a long history of medicinal use in Ayurveda and Chinese medicine. Even though the leaves of this plant are used in diabetes mellitus, there have been no in vivo studies to prove its efficacy. The aim of this study was to know the efficacy of ethanol extract of T. foenum graecum leaves on blood glucose levels, antioxidant enzymes, islets cells of pancreas, creatinine and urea levels in normal and streptozotocin induced diabetic rats. Diabetes was induced by streptozotocin (45 mg/kg b.w. in 0.9 % cold saline). Two doses (250 and 500 mg/kg b.w.) of the extracts were administered in the study. The activity was compared with the reference standard glibenclamide (0.5 mg/kg b.w.) for various biochemical and histopathological parameters. The data was analysed by one way ANOVA followed by Turkey's post hoc test. The activity of the extract in reducing blood glucose, creatinine and urea levels, in enhancing antioxidant enzyme activity and restoring and regenerating islet cells of pancreas was comparable to glibenclamide. The result suggests that ethanol leaf extract of T. foenum graecum possesses significant antidiabetic property.

Keywords Diabetic rats · Trigonella foenum graecum · Antihyperglycemic effect · Antioxidant enzyme activity

R. Premanath (*) : N. Lakshmidevi
e-mail: rmy300@yahoo.co.in

Introduction

Diabetes Mellitus (DM) is a leading metabolic disorder worldwide, caused by a deficiency in the production of insulin in the beta cells of pancreas, or by the insulin resistance. Such a deficiency results in the increased concentration of glucose in the blood which results in secondary complications affecting eyes, kidneys, nerves and arteries. Experimental evidences suggest the involvement of free radicals in the pathogenesis of diabetes [1] and more importantly in the development of diabetic complications [2].

Trigonella foenum graecum Linn. commonly known as Fenugreek belongs to the family Fabaceae, which is an annual, herbaceous and aromatic plant. It is one of the oldest medicinal plants, originating in India and Northern Africa. The leaves and seeds, which mature in long pods, are used to prepare extracts or powders for medicinal use. In India, fenugreek seeds are commonly consumed as a condiment. Fresh and dried fenugreek leaves and tender stems are edible which are widely used as a vegetable. Fenugreek

is reported to have anti-diabetic, anti-fertility, anticancer, anti-microbial, anti-parasitic, lactation stimulant and hypocholesterolemic effects [8].

In view of the promising anti-diabetic potential of fenugreek seeds, we have investigated the efficacy and safety of ethanol extract of its leaves on blood glucose levels, its effect on kidney parameters, antioxidant enzyme activity in liver and islet cells of pancreas in streptozotocin induced diabetic rats.

Material and Methods

Plant material

Fresh and healthy leaves of T. foenum graecum were obtained from local market. The sample specimen was identified based on the taxonomical characteristics and deposited in the herbarium of department of Applied Botany. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 40 ° C for 48 h, powdered and used for extraction.

Preparation of solvent extract

Fifty grams of the powdered material was extracted initially with 300 ml of ethanol for 24 h at 23±2 ° C. The extract was filtered with sterile Whatman filter paper into a clean conical flask. Second extraction was carried out with same amount of solvent for another 24 h at 23±2 ° C and filtered. The extracts were later pooled and transferred into the sample holder of the rotary flash evaporator for the evaporation of the solvents. The evaporated extract so obtained was preserved at 4 ° C in airtight bottle until further use [9]. The suspension of the extract was prepared by dissolving the weighed amount in freshly prepared 1 % gum arabic solution.

Animals

Adult healthy Albino rats of Wistar strain of either sex weighing 120–160 g with no prior drug treatment were used in the present study. Animals were maintained at 22±2 ° C with 12 h light and dark cycle. The animals were fed on standard pellet diet and had free access to diet and water throughout the experiment. Animals that are described as fasting were deprived of food for at least 16 h but were allowed free access to drinking water. Animal study was performed in Central Animal Facility, JSS Medical College, Mysore, with due permission from Institutional Animal Ethics Committee (R.No. JSS/MC/IEC/5064/CPCSEA).

Experimental design

The animals were randomly divided into the seven groups with six animals in each group. Group A served as saline control, group B and C served as normal controls who were administered 250 and 500 mg /kg body weight (b.w.) of leaf extract respectively to know the toxic effects of the extracts if any on kidney and liver, group D as the diabetic control, Group E as the standard drug (glibenclamide, 0.5 mg/kg b.w.) control and group F and G were diabetics treated with 250 and 500 mg /kg b.w. of leaf extract respectively. Saline, leaf extract and the standard drug were administered orally using a mouth gauge. During the study period, body weight, food and water intake of rats were monitored regularly.

After initial determination of 16 h fasting blood glucose levels (blood drawn through the tail vein puncture) animals were given single intraperitoneal injection of streptozotocin at a dose of 45 mg/kg b.w. freshly dissolved in cold 0.9 % saline [10]. Fasting blood glucose levels were recorded after 5 days. Animals that developed stable hyperglycemia with fasting blood glucose levels more than 200 mg/dl were selected for the study.

Rats were fasted overnight; blood was drawn from the tail by tail vein puncture during the experimental period and from cardiac puncture at the end of the experimental period (after 28 days) under anaesthesia. Serum was separated by centrifuging the collected blood samples at 10,000 rpm for 10 min at 4 ° C and used for the estimation of creatinine and urea.

Urine from normal control rats, diabetic and treated diabetic rats were collected under a layer of toluene by keeping the rats in metabolic cages for 24 h. Collected urine samples were filtered through filter paper, centrifuged and stored at 4 ° C until further analysis.

Preparation of liver tissue homogenate

After 28 days, rats were dissected under anaesthesia. Liver was rinsed in ice cold distilled water followed by chilled 0.9 % saline. About 1 g of liver tissue was homogenized in 10 ml of 10 mM phosphate buffered saline (pH 7.4) using REMI homogenizer fitted with a teflon plunger. The homogenate was centrifuged at 10,000 rpm at 4 ° C for 15 min and the supernatant was used for the determination of antioxidant liver enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px).

Analytical Methods

Fasting blood glucose was measured by glucose oxidase-peroxidase (GOD-POD) method in mg/dl using a digital glucometer (Braun Omnitest^{R} EZ, Germany) [11]. Creati- nine estimation was carried out in both serum and urine samples [12]. Blood urea was estimated by enzymatic UV- kinetic method [13]. The activity of SOD was assayed in a Daytona analyser using commercially available diagnostic SOD kit [14]. The activity of catalase was assayed based on the hydrogen peroxide decomposition method [15]. The activity of glutathione peroxidase was assayed in an auto- matic analyser using glutathione peroxidase kit [16].

Histopathological studies

At the end of the study, the rats were sacrificed, whole pancreas from each animal was removed, washed in normal saline and fixed in 10 % formalin, embedded in paraffin and sections of 3–5 μ thickness were cut and routinely stained with basic dye hematoxylin and acidic dye eosin to differentiate the nucleus and cytoplasm. The sections were studied for the islet cell characteristics using a binocular compound microscope.

Statistical analysis

All the values were expressed as mean ± standard error of mean (SEM). Statistical difference was evaluated by using one way analysis of variance (ANOVA) followed by Turkeys post hoc test. Data were considered statistically significant at P value≤0.05.

Results

Effect of leaf extract on blood glucose level

The anti-hyperglycemic effect of the extract on the fasting blood glucose levels of diabetic rats is shown in Table 1. Administration of streptozotocin increased the blood glucose level by 68.7 % in diabetic control rats which was maintained over a period of 4 weeks. Daily treatment of ethanol extract of T. foenum graecum leaves at a concentration of 250 and 500 mg/kg b.w. for a period of 28 days led to a dose dependent fall in the blood glucose levels by 23.5 % (P00.000) and 31.2 % (P00.000) respectively. The glucose lowering effect of the extract seems to reach a maximum after 25 days of treatment and remained almost constant during the next 4–5 days. The effect was more pronounced with animals receiving 500 mg/kg b.w. of extract which can be compared to that of glibenclamide. The fasting glucose levels in normal control rats and normal control rats fed with the extracts were found to be stable all throughout the experimental period.

Effect of leaf extract on body weight

Prior to streptozotocin administration, there was no significant difference in the average body weight of all the seven groups of experimental animals. By the end of the first week after DM was experimentally induced, the weights of group D, E, F, and G were significantly reduced as shown in Table 2. This weight loss continued for 4 weeks in diabetic control animals (36.3 %). However, the weight of animals in groups F and G increased by 42 % (P00.000) and 44.9 % (P00.000) on treatment with 250 and 500 mg/kg b.w. of T. foenum graecum leaf extract. At the end of the experimental period there was a significant difference in the weights of groups F and G as compared to group D. Animals in groups A, B and C showed an increase in their body weights during the experimental period.

Effect on kidney parameters

The effect of T. foenum graecum leaf extract on serum and urinary creatinine and serum urea is shown in Table 3. Diabetic control rats exhibited higher serum creatinine, urinary creatinine and blood urea levels compared to those of normal rats. The creatinine and urea levels were significantly decreased by glibenclamide and the extract by 28 days of treatment. The extract at a concentration of 500 mg/kg b.w. showed a greater decrease in serum creatinine (73.62 %, P00.000), urinary creatinine (62.7 %, P0 0.000) and serum urea (50.6 %, P0 0.000) levels which was even better than glibenclamide treated rats. There were no changes in the creatinine and urea levels in normal extract treated controls (group B and C) as compared to the normal control animals.

Effect on liver antioxidant enzymes

As shown in Table 4, streptozotocin induced diabetic control rats showed a decrease in SOD (3.23 U/mg protein), catalase (6.37 U/min/mg protein) and GSH-Px (3.45 U/mg protein) as compared to the normal rats. Administration of T. foenum graecum leaf extract for 28 days produced a marked increase in the enzyme activities in both F and G groups. There was a greater increase in the activity of SOD (14.7 %, P00.000) in diabetic rats treated with 500 mg/kg b.w. of extract when compared to glibenclamide treated rats. There was an increase in the enzyme activities in group B and C animals also.

Table 1 Effect of ethanol extract of Trigonella foenum graecum leaves on glucose level in normal and streptozotocin induced diabetic rats (N0 6)

Groups	Fasting blood glucose levels (mg/dl)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Group A Normal control (0.9 % NaCl)	77.0±1.82	78.25±0.95	77.75±2.21	79.50±2.64	79.25±1.70
Group B Normal rats with extract (250 mg/kg b.w)	78.75±2.62	78.0±3.16	78.50±2.88	79.25±2.21	79.50±1.29
Group C Normal rats with extract (500 mg/kg b.w)	78.50±3.10	79.25±2.50	78.75±2.50	79.75±2.98	78.25±2.75
Group D STZ induced diabetic control	246.25±9.53*	284.0±5.47*	309.50±8.73*	319.75±7.80*	319.50±8.66*
Group E STZ+ glibenclamide (0.5 mg/kg b.w)	248.50±13.52	222.2±7.27**	190.0±4.54**	178.25±6.80**	157.75±8.95**
Group F STZ+ extract (250 mg/kg b.w)	248.25±10.65	240.0±12.27**	226.50±8.58**	208.5±7.41**	189.75±6.39**
Group G STZ+ extract (500 mg/kg b.w)	243.25±12.03	229.0±11.40**	204.0±6.21**	184.50±5.44**	167.25±7.80**

Values are expressed as mean ± S.E.M.; *- significantly different from normal control (P≤0.05); **- significantly different from diabetic control (P≤0.05)

Histopathological studies

As shown in Fig. 1, in the diabetic group, degeneration with reduction in the number and reduced dimensions of the islets were observed in the pancreas. In animals treated with 250 mg/kg b.w. of the extract, the restoration of the normal cellular population and size of islets were noted especially in the central β cell region. In animals treated with 500 mg/kg b.w. of the extract, there was not only an increase in the cellular population and size of the islets but also there was an increase in the number of islets noted. The regeneration noted in the β cell regions was comparable to that noted with glibenclamide.

Discussion

Streptozotocin, N-[methylnitrocarbamoyl]-D-glucosamine, is a potent methylating agent for DNA and acts as nitric oxide donor in pancreatic cells. β cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of free radical scavenging enzymes [17]. For the study of antihyperglycemic agents, streptozotocin induced hyperglycemia in rodents is considered to be a good preliminary screening model [18].

Streptozotocin induced diabetes produced a significant increase in glucose levels. Treatment with ethanol extract of T. foenum graecum leaves showed significant decrease in glucose levels which was near to glibenclamide treated control. Decrease in blood glucose levels were found to be more effective with 500 mg/kg b.w. of extract which showed its dose dependent effects. Glibenclamide showed rapid normalization of blood glucose due to its insulin releasing effects. Earlier studies by various workers have shown the antihyperglycemic activity of T. foenum graecum seeds in a dose dependent manner in experimentally induced diabetic rats [19, 20]. The anti-diabetic plant extracts may involve one or more compounds which decrease blood glucose suggesting that the natural constituents could act synergistically to induce a hypoglycemic effect [21].

Induction of diabetes with streptozotocin is associated with a characteristic loss of body weight, which is probably due to muscle wasting [22]. In our study, there was a

Table 2 Effect of treatment of Trigonella foenum graecum leaf extract on body weight of normal and streptozotocin induced diabetic rats (N0 6)

Groups	Average body weight (g)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Group A Normal control rats (0.9 % NaCl)	142.50±5.0	146.25±2.50	152.50±5.0	160.0±4.08	166.25±4.78
Group B Normal rats with extract (250 mg/kg b.w)	140.0±0.0	145.0±4.08	153.75±4.78	158.75±4.78	163.75±4.71
Group C Normal rats with extract (500 mg/kg b.w)	140.0±8.16	146.25±4.78	153.75±7.50	161.25±8.53	165.0±9.12
Group D STZ induced diabetic control	135.50±5.77	115.0±5.77*	105.0±5.77*	95.0±4.08*	86.25±4.78*
Group E STZ+ glibenclamide (0.5 mg/kg b.w)	132.5±05.0	120.0±8.16**	125.0±5.77**	123.75±4.78**	126.25±2.50**
Group F STZ+ extract (250 mg/kg b.w)	127.5±05.0	117.50±2.88**	116.25±4.78**	120.0±4.08**	122.5±2.88**
Group G STZ+ extract (500 mg/kg b.w)	133.75±4.78	121.25±2.50**	123.75±2.50**	124.50±4.20**	125.0±4.08**

Values are expressed as mean ± S.E.M.; *- significantly different from normal control (P≤0.05); **- significantly different from diabetic control (P≤0.05)

Table 3 Effect of treatment of Trigonella foenum graecum leaf extract on serum creatinine, urinary creatinine and serum urea levels in normal and streptozotocin induced diabetic rats (N0 6)

Groups	Treatment	Serum creatinine (mg/dl)	Urinary creatinine (mg/day)	Serum urea (mg/dl)
A	Normal control rats (0.9 % NaCl)	0.47±0.04	18.72±1.83	23.74±0.73
B	Normal rats with extract (250 mg/kg b.w)	0.41±0.08	18.72±0.62	23.62±0.79
C	Normal rats with extract (500 mg/kg b.w)	0.36±0.05*	18.32±0.36	23.42±0.74
D	STZ induced diabetic control	1.82±0.17*	51.52±3.76*	61.94±1.54*
E	STZ+ glibenclamide (0.5 mg/kg b.w)	0.60±0.07**	23.61±2.38**	29.73±0.59**
F	STZ+ extract (250 mg/kg b.w)	0.50±0.03**	22.22±2.30**	32.63±0.29**
G	STZ+ extract (500 mg/kg b.w)	0.48±0.01**	19.17±0.84**	30.55±0.44**

Values are expressed as mean ± S.E.M.; *- significantly different from normal control (P≤0.05); **- significantly different from diabetic control (P≤0.05)

significant weight loss in the diabetic rats, whereas treatment with the ethanol extract of leaves of T. foenum graecum at both the doses showed improvement in their body weight, indicating that the extract had beneficial effect in preventing loss of body weight of diabetic rats. The probable mechanism is due to its effect in controlling muscle wasting [23]. The metabolic disorders were corrected after the administration of plant extract for 28 days as shown by reduction in polyphagia, polyuria and polydipsia in diabetic rats treated with plant extract. This could be the result of improved glycemic control produced by ethanol extract of T. foenum graecum.

Diabetic nephropathy is the leading cause of DM related morbidity and mortality. The pathogenesis of diabetic nephropathy is related to chronic hyperglycemia and hemodynamic alterations in renal microcirculation and structural changes in glomerulus as evident by the significant elevation in creatinine and urea levels [24]. Elevated creatinine and urea levels were significantly decreased in diabetic rats treated with T. foenum graecum extract which indicated its beneficial effects on kidney. The result indicate that the ethanol extract of T. foenum graecum leaves does not alter the kidney function, as it is evident from the normal creatinine and urea levels in normal control animals treated with the extract (both 250 and 500 mg/kg b.w.) for a period of 28 days.

Diabetes is usually accompanied by increased production of free radicals [25]. Free radicals are capable of damaging cellular molecules, DNA, proteins and lipids leading to altered cellular functions [26]. Abnormally high levels of free radicals and simultaneous decline of antioxidant defence mechanisms can lead to increased lipid peroxidation and development of insulin resistance [27]. In the present study, diabetic control rats showed significant decrease in SOD, catalase and GSH-Px levels in rat liver homogenate compared to normal rats, indicating dysfunction in antioxidant defensive system. Treatment with ethanol extract of T. foenum graecum leaves increased SOD, catalase and GSH-Px levels in diabetic rat liver homogenates which were comparable to that of glibenclamide treated rats. Vitamin C and β carotene have been shown to be present in T. foenum graecum leaves [28]. Effect of Vitamin C, E and β carotene in elevating the antioxidant enzyme activities in diabetic rats has been proved [29]. Phenolics, which are the potential antioxidants are also present in T. foenum graecum leaves [30]. So, the activity of the extract in the present study is probably due to the presence of Vitamin C, β

Table 4 Effect of ethanol extract of Trigonella foenum graecum leaves on liver antioxidant enzymes in normal and streptozotocin induced diabetic rats (N0 6)

Groups	Treatment	Superoxide dismutase (units/mg protein)	Catalase (units/min/mg protein)	Glutathione peroxidase (units/mg protein)
A	Normal control rats (0.9 % NaCl)	10.50±0.54	10.70±1.13	5.73±0.28
B	Normal rats with extract (250 mg/kg b.w)	12.39±0.75*	11.12±0.40	6.08±0.18
C	Normal rats with extract (500 mg/kg b.w)	14.15±0.26*	11.35±0.42	6.30±0.24*
D	STZ induced diabetic control	3.23±0.30*	6.37±0.48*	3.45±0.36*
E	STZ+ glibenclamide (0.5 mg/kg b.w)	10.02±0.33**	8.82±0.63**	5.26±0.27**
F	STZ+ extract (250 mg/kg b.w)	10.08±0.43**	7.52±0.61**	4.93±0.16**
G	STZ+ extract (500 mg/kg b.w)	11.50±1.26**	8.32±0.40**	5.48±0.17**

Values are expressed as mean ± S.E.M.; *- significantly different from normal control (P≤0.05); **- significantly different from diabetic control (P≤0.05)

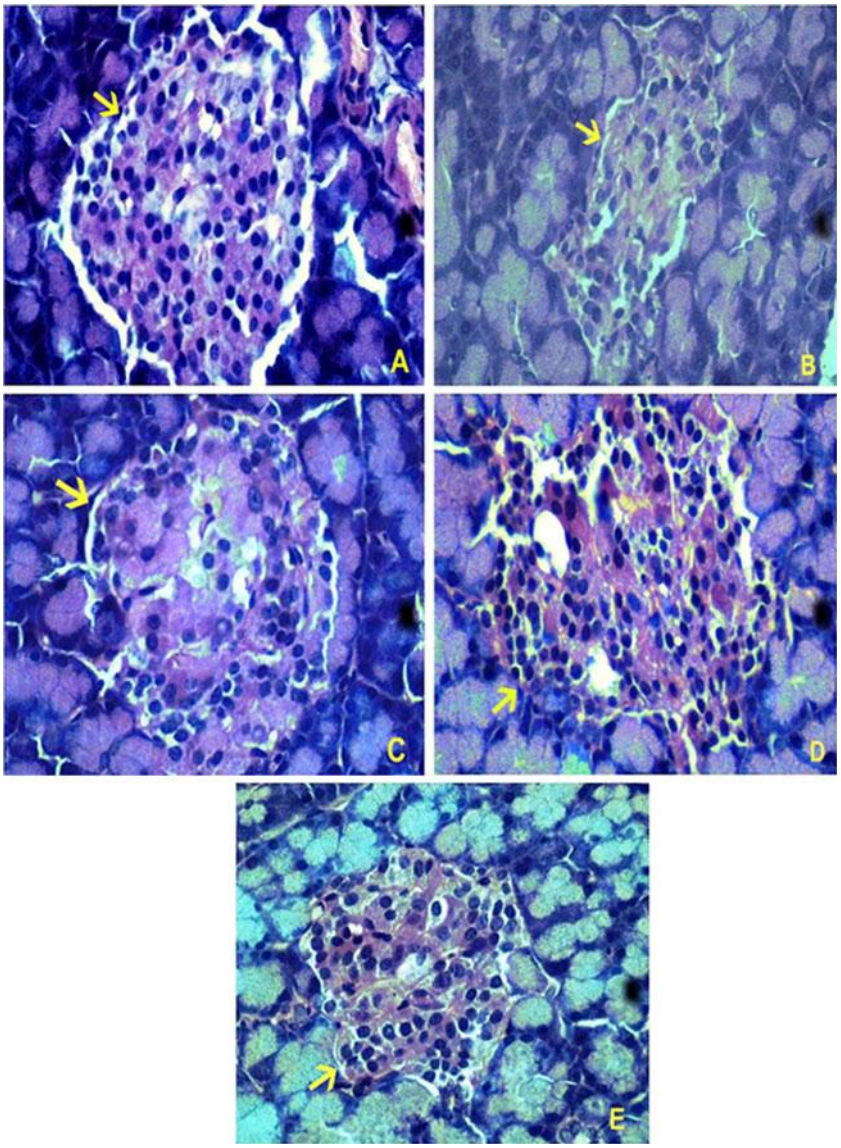

Fig. 1 Photomicrographs of rat pancreas stained by H and E stain of normal a and diabetic b rats and effects of glibenclamide c, 250 mg/kg b.w. ethanol extract d and 500 mg/kg b.w. ethanol extract e of Trigonella foenum graecum. Microscope magnification (40x)

carotene or phenolic antioxidant constituents. An increase in the liver antioxidant enzymes in non diabetic control rats treated with the leaf extract of T. foenum graecum showed their non-toxic nature on the liver. The improvement in the enzyme activities in non-diabetic control rats with extracts when compared with the non diabetic control rats without extracts is indicative of better liver function. The result of our study is in agreement with the earlier study on fenugreek seeds [31].

Degeneration and necrosis of the islets of pancreas in diabetic rats was observed similar to the observation of Selvan [32]. Our study has shown the regeneration of islet cells especially in the β cell region. Similar effects in streptozotocin treated diabetic animals were reported [33, 34]. The effect of the plant extract in regeneration in the β cell region might be due to the presence of β carotene [35]. Photomicrographical data in our studies reinforce the healing of pancreas, by T. foenum graecum leaf extract as a plausible mechanism of their antidiabetic activity.

In conclusion, Our data suggest that the ethanol leaf extract of T. foenum graecum possess significant antidiabetic activity as it lowers blood glucose levels in diabetic rats. It also increases body weight of diabetic rats. The ethanol extract was seen to be effective in preventing the rise of creatinine and urea levels and thus improve the kidney functions which were impaired in the streptozotocin induced diabetic rats. In addition to this, the extract was not found to exert any toxic side effects in the extract-fed normal rats as seen by the normal creatinine and urea levels. The extract has shown to induce regeneration and restoration of the normal morphology of the islets of pancreas. Along with the antihyperglycemic activity, the extract was found to possess potential antioxidant activity and enhanced antioxidant status of the diabetic rats. The present study

suggests that the leaves of T. foenum graecum can be utilized for the management of diabetes, but further studies on the nature of active principles involved and their mechanism of action need to be studied.

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Conflicting interest None

References

1. Matteucci E, Gimapietro O. Oxidative stress in families of type I diabetic patients. Diabetes Care. 2000;23:1182–6.
2. Lipinski B. Pathophysiology of oxidative stress in diabetes mellitus. J Diabetes Complic. 2001;15:201–10.
3. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract.
2010;87:4–14.
4. Ramachandran A, Snehalatha C, Viswaanthan V. Burden of type 2 diabetes and its complications-the Indian scenario. Curr Sci.
2002;83:1471–6.
5. Hussain AMHE. Hypoglycemic, hypolipidemic and antioxidant properties of combination of Cucurmin from Cucurma longa Linn. And partially purified product from Abroma augusta Linn. In streptozotocin induced diabetes. Indian J Clin Biochem.
2002;17:33–43.
6. Grover JK, Yadav S, Vats V. Medicinal plants of India with antidiabetic potential. J Ethnopharmacol. 2002;81:81–100.
7. Modak M, Dixit P, Londhe J, Ghaskadbi S, Devasagayam TPA. Indian herbs and herbal drugs used for the treatment of Diabetes. J Clin Biochem Nutr. 2007;40:163–73.
8. Al-Habori M, Raman A. Pharmacological Properties in Fenugreek. In: Petropoulos GA, editor. The Genus Trigonella. London and New York: Taylor and Francis; 2002. p. 163–82.
9. Okigbo RN, Mbajiuka CS, Njoku CO. Antimicrobial potentials of Xylopia aethopica and Occimum gratissimum L. on some pathogens of man. Int J Mol Med Adv Sci. 2005;1:393–7.
10. Venkateswaran S, Pari L. Effect of Coccinia indica leaves on antioxidant status in streptozotocin induced diabetic rats. J Ethnopharmacol. 2003;84:163–8.
11. Trinder P. Determination of blood glucose using an oxidase peroxidase system with a non carcinogen chromogen. J Clin Pathol. 1969;22:158–61.
12. Folin O, Wu H. A system of blood analysis. J Biol Chem. 1919;38:81–110.
13. Talke H, Schubert GE. Enzymatic urea determination in the blood and serum in the warburg optical test. Wien Klin Wochenschr.
1965;43:174–75.
14. Woolliams JA, Wiener G, Anderson PH, Mc Murray CH. Variation in the activities of glutathione peroxidise and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. Res Vet Sci. 1983;34:253–6.
15. Luck H. Catalase: methods of enzymatic analysis. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic; 1971. p. 885–93.
16. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidise. J Lab Clin Med. 1967;70:158–69.
17. Spinas GA. The dual role of nitric oxide in islets β cells. News Physiol Sci. 1999;14:49–54.
18. Ivorra MD, Paya M, Villar A. A review of natural products and plants as potential antidiabetic drugs. J Ethnopharmacol. 1989;27:243–75.
19. Vats V, Grover JK, Rathi SS. Evaluation of antihyperglycemic and hypoglycemic effect of Trigonella foenum graecum Linn, Ocimum sanctum Linn and Pterocarpus marsupium Linn. In normal and alloxanized diabetic rats. J Ethnopharmacol. 2002;79:95–100.
20. Xue WL, Li XS, Zhang J, Liu YH, Wang ZL, Zhang RJ. Effect of Trigonella foenum graecum (fenugreek) extract on blood glucose, blood lipid and hemorrheological properties in streptozotocin induced diabetic rats. Asia Pac J Clin Nutr. 2007;16:422–6.
21. Roy S, Sehgal Padhy BM, Kumar VL. Antioxidant and protective effect of Calatropis procera against alloxan induced diabetes in rats. J Ethnopharmacol. 2005;102:470–3.
22. Swanston-Flatt SK, Day C, Bailey CJ, Flatt PR. Traditional plant treatment for diabetes: Studies in normal and streptozotocin dia- betic mice. Diabetologia. 1990;33:462–4.
23. Whitton PD, Hems DA. Glycogen synthesis in perfused liver of streptozotocin diabetic rats. Biochem J. 1975;21:150–3.
24. Cryer PE. Hypoglycemic agents. In: Braunwald E, Fanci AS, Kasper DL, Hanser SL, Longo DL, Jameson JL, (eds). Harrison's Principles of Internal Medicine, 15th ed., vol 2. Mc Graw-Hill; 2001: 2138–2143.
25. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress and antioxidants: A review. J Biochem Mol Toxicol. 2003;17:24–38.
26. Oberlay LW. Free radicals and diabetes. Free Radic Biol Med. 1988;5:113–24.
27. Garg MC, Bansal DD. Protective antioxidant effect of vitamin C and E in streptozotocin induced diabetic rats. Indian J Exp Biol. 2000;38:101–4.
28. Srinivasan K. Fenugreek (Trigonella foenum graecum): A review of health beneficial physiological effects. Food Rev Int. 2006;22:203–24.
29. Mekinova D, Chorvathova V, Volkovova K, Staruchova M, Grancicova E, Klvanova J, Ondreicka R. Effect of intake of exogenous vitamin C, E and β carotene on the antioxidative status in kidneys of rats with streptozotocin induced diabetes. Nahr. 1995;39:257–61.
30. Ramya P, Sudisha J, Lakshmidevi N, Aradhya SM. Antibacterial and anti-oxidant activities of fenugreek (Trigonella foenum graecum L.) Leaves. Res J Med Plant. 2011;5:695–705.
31. Muralidhara, Narasimhamurthy K, Viswanatha S, Ramesh BS. Acute and subchronic toxicity assessment of debitterized fenugreek powder in the mouse and rat. Food Chem Toxicol. 1999;37:831–8.
32. Selvan VT, Manikandan L, Senthil Kumar GP, Suresh R, Kakoti BB, Gomathi P, Kumar DA, Saha P, Mazumder UK. Antidaibetic and antioxidant effect of methanol extract of Artanema sesamoides in strep- tozotocin induced diabetic rats. Int J Appl Res Nat Prod. 2008;1:25–33.
33. Shanmugasundaram ER, Gopinath KL, Radha KS, Rajendran VM. Possible regeneration of the islets of langerhans in streptozotocin diabetic rats given Gymnema sysvestre leaf extracts. J Ethnopharmacol.
1990;30:265–79.
34. Xiu LM, Miura AB, Yamamoto K, Kobayashi T, Song QH, Kitamura H, Cyong JC. Pancreatic islet regeneration by ephedrine in mice with streptozotocin induced diabetes. Am J Chin Med. 2001;29:493–500.
35. Nagappa AN, Thakurdesai PA, Venkat Rao N, Jiwan Singh M. Antidiabetic activity of Terminalia catappa Linn. Fruits. J Ethnopharmacol. 2003;88:45–50.

ORIGINAL ARTICLE

Cardiometabolic risk, insulin resistance and immunity in HIV/AIDS patients receiving highly active retroviral therapy

Kofoworola Awotedu & Benjamin Longo-Mbenza & John Sungwacha Nasila & Abolade Awotedu & Chukwuma Ekpebegh

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Abstract Prior to the advent of HAART (Highly Active retroviral therapy), studies of insulin resistance in HIV infection showed normal insulin sensitivity. The metabolic disturbances due to HAART are now reported in our setting. To investigate potential associations between cardiometabolic indices, innate and adaptive immunity, adipokines, and markers of insulin resistance (IR) in HIV/AIDS patients on first-line antiretroviral therapy (ART) were studied. This cross-sectional study selected HIV/AIDS patients treated with ART at public clinics of Mthatha Municipality, South Africa. Age, waist-to-hip ratio, blood pressure, total cholesterol (TC), HDL-C, LDL-C, triglycerides, C-peptide, uric acid, C-reactive protein (CRP), glucose, and albuminuria were the cardiometabolic indices. Innate immunity (lymphocytes), adaptive immunity (CD4 cell count), adipokines (adiponectin, leptin), and insulin resistance markers—fasting serum Insulin and homeostasis model assessment—IR score were also explored. Out of 258 selected patients, aged 38.1±9.3 years, 79.5 % were females. There was a univariate correlation between HOMA-IR score and triglyceride (r00.331; P<0.0001), uric acid (r00.386; P00.008), albuminuria (r00.316; P<0.0001), adiponectin (r0−0.322; P00.035), lymphocytes (r00.186; P00.04) and waist-to-hip ratio (r00.217; P00.013), and HOMA-IR score. C-peptide was correlated with serum Insulin (r00.694; P<0.0001). After adjusting for confounding factors, only triglycerides and C-Peptide independently and significantly explained 64.1 % of the variance in HOMA-IR score. Patients receiving ART are at higher risk for diabetes mellitus, cardiovascular disease and chronic renal disease. Regular monitoring of patients on first-line anti-retroviral agents is imperative to detect these cardiometabolic risk factors and allow for treatment to be initiated early.

Keywords Insulin resistance · Metabolic syndrome · Diabetes · Cardio-renal diseases · Antiretroviral therapy

B. Longo-Mbenza (✉)
longombenza@gmail.com

Introduction

Prior to the advent of highly active antiretroviral therapy (HAART), studies in HIV infected patients showed normal insulin sensitivity [1]. Hypertriglyceridemia was the only metabolic abnormality reported in advanced HIV disease (AIDS) which was attributed to high levels of cytokines accompanying chronic infection [2].

Since the introduction of HAART in 1995, there has been major reduction in the morbidity and mortality from HIV/AIDS. It is now recognised as a chronic manageable disease for many patients worldwide [3]. Consequently the resulting increase in survival while on HAART exposes HIV positives to the cumulative toxicity of the drugs. This results in higher risks for diseases like the metabolic syndrome, diabetes mellitus (DM), arterial hypertension, hyperuricemia, microalbuminuria, renal and cardiovascular events [4–11] associated with Insulin Resistance (IR). Surrogate markers thus include abdominal obesity, DM and cardiovascular

diseases (CVD), and are often associated with the metabolic syndrome (Mets) [12]. IR has been defined as a state where a greater than normal level of insulin is necessary to obtain a quantitatively physiological response [13]. The gold standard tests for measuring IR is the hyperinsulinemic euglycemic clamp. This test is expensive, technically difficult and time consuming. Alternatively, the homeostasis model assessment of (HOMA) of IR [13–15] is easier to apply in large epidemiological data as it relies on only the fasting plasma glucose and fasting insulin levels without the need for hyperinsulinemic euglycemic clamp study.

A recent study by us showed that low HDL-cholesterol (HDL-C) and exposure to HAART without protease inhibitors were the independent determinants of IR in black patients from Eastern Cape province of South Africa [15].

Increase in microalbuminuria (systemic endothelial dysfunction and inflammation) [17–19], C-reactive protein (inflammation), triglycerides, C-peptide [20], uric acid [21], and chronic activation of the innate immune system (leucocytes, lymphocytosis) [22, 23] are all related to the metabolic syndrome/insulin resistance and atherosclerosis.

The objective of the present study was to examine the relationships between anthropometric parameters, blood lipids/lipoproteins, uric acid, urea, C-peptide, lymphocytes, adiponectin, albuminuria, and HOMA –IR in HAART treated patients.

Methods

This was a cross sectional study conducted between September 2009 and June 2010 at the following Public health Clinics in Mthatha, Oliver Tambo district of the Eastern Cape Province of South Africa (Infectious disease Clinic of Mthatha Hospital Complex, The Gateway Clinic, and the Stamford Terrace Clinic). The study population is comprised of HIV/AIDS patients receiving antiretroviral therapy such as non-nucleoside reverse transcriptase inhibitors (NNRTI) and nucleoside reverse transcriptase inhibitors (NRTI) which are the first-line drugs used in most public hospitals in the Eastern Cape of South Africa (SA). The drugs mostly used are Lamivudine (3TC), Stavudine (D4T) or Azathioprin (AZT), and Efavirenz or Nevirapine. Study protocol was approved by the Ethics committee of Walter Sisulu University School of Medicine and the study was conducted according to the Helsinki Declaration [16]. Writ- ten informed consent was obtained from each patient. Struc- tured and standardized questionnaires were administered to the patients to obtain demographics (age and gender) and antiretroviral therapy duration. Weight was measured to the nearest 0.1 kg with patients barefooted and wearing only light clothing using monitor BF500 (Omron Inc, Tokyo, Japan). The height of each patient was taken to the nearest
1 mm with the aid of Harpenden Stadiometer (Drinago Trading, Amsterdam, Netherlands) with the patients barefooted, standing straight with the feet together. Body mass index (BMI), total body fat, skeletal muscle fat, and visceral fat were obtained using Omron body composition monitor 500 (Omron Japan). These values were automatically obtained after entering the age, gender, and height of the patient into the machine. Waist measurement was measured to the nearest 1 cm at the level midway between the lower rib margin and the iliac crest and hip measurement to the nearest 1 cm at the maximal girth over the buttocks using a non-elastic standard tape. To determine the serum levels of adiponectin (mg/ml), serum leptin (mg/ml), serum insulin (μU/ml), serum C-reactive protein (CRP), serum total cholesterol (TC in mmol/l), high density lipoprotein cholesterol (HDL-C in mmol/l) serum Triglyceride (mmol/l), C-peptide (μg/ml), serum Creatinine (μmol/l), serum Uric acid (μmol/l) , serum urea (mmol/l) and plasma glucose. Twenty milligrams of blood was drawn from the right antecubital vein of each patient at 7:30 AM before breakfast after an overnight fast of 12 h. The blood samples were analysed the same day in the National Health Laboratory Services (NHLS) in Mthatha, SA. Adiponectin and leptin were determined using the Quantitative kit high sensitivity and the quantitative sandwich radioimmuno-Assay (RIA) (R&D systems, Minneapolis, USA) which is made up of 96 plate layout. The routine biochemistry parameters were measured using an automated analyzer (Hitachi/Cobas C system. Tokyo, Japan). Full blood count (mm^3) including neutrophils, monocytes, lymphocytes and platelets was performed using an automated counter (Coulter Model Sysmex XT 2000, Sysmex Corporation, Kobe, Japan). CD4 lymphocyte cell count /L was determined by flow cytometry using FC 500 MPL equipment (Beckman Coulter, Brea, CA, USA). Serum CRP was measured using a commercial kit assay (Behring diagnostics, Marburg, Germany). Microalbuminuria was estimated by an immunoturbidometric method on the Roche Cobas Mira analyzer (Roche Diagnostics, Basel, Switzerland) in mid stream urine collected after cleaning the private parts thoroughly. Patients with urinary tract infection and menstruating women were excluded.

HOMA-IR score (index) was calculated as follows:[Fasting insulin in μU /ml X fasting glucose in mmol/l / 22.5] [13]. Increase in microalbuminuria (systemic endothelial dysfunction and inflammation) [17–19], C-reactive protein (inflammation), triglycerides, C-peptide [20], uric acid [21], and chronic activation of the innate immune system (leucocytes, lymphocytosis) [22, 23] are all related to the metabolic syndrome/insulin resistance and atherosclerosis.

Statistical analysis

Continuous variables were expressed as means ± standard deviations, gender (qualitative variable) was

presented as proportions (%) of males and females and as sex ratio.

In univariate (or bivariate) analyses, we used Pearson correlation analysis (r coefficient) to assess the association of insulinemia or HOMA-IR score with the rest of the study variables. A multivariate linear regression model was used to determine the variations (determination R^2 coefficient) of HOMA–IR score as a function (dependent variable) of its significant correlates from univariate analysis and after adjusting for confounding factors. A P-value < 0.05 was considered as statistically significant. Increase and decrease in levels of variables were defined by the direction of the correlation coefficients. All statistical analyses were performed using Statistical Package for Social Sciences SPSS/PC+) version 18.0 for Windows (SPSS Inc, Chicago.Il.USA).

Results

Two hundred and fifty eight HIV infected participants on Ist line antiretroviral (53 males 020.5 % and 205 females 079.5 %) with a sex ratio of 4 females: 1 male, were analyzed. The mean ± SD (range) of age, HAART duration, CD4 count, viral load, neutrophils, monocytes, platelets and lymphocytes in the study samples were 38.1±9.3 years (19–62 years) 24±12 months, 367.2±230.2 cells/ul (4–1400), 21646.3±12930.7 copies/ml (5674.1–54668 copies/ml), 50.9±9.8 % (29.5–70.1 %), 8± 2.7 % (1.4–14 %), 259800±79400/mm^3(10000–534000) and 36.6±9.3 % (17–56 %), respectively

Table 1 with mean levels of the traditional and new cardiovascular risk factors shows mean BMI in the overweight range and mean LDL and triglyceride levels higher than recommended levels. Mean HOMA-IR was in the insulin sensitive range.

Table 2 shows univariate correlates of serum insulin. The increase in serum insulin was associated more with the increase in serum C-peptide, serum uric acid, and albuminuria than with that of lymphocytes and waist to hip ratio respectively.

Table 3 shows univariate correlates of HOMA-IR score. The decrease in adiponectin concentration and the increase in levels of triglycerides, uric acid, urinary albumin, C-peptide, lymphocyte count and waist to hip ratio determined a significant increase in the levels of HOMA–IR score, respectively. However after entering the later variables onto a multivariate linear regression, only individual increase in serum C- peptide and serum triglycerides were significantly associated with increase (adjusted R^2 064.1 % variations) of HOMA-IR score after adjusting for gender, age, serum uric acid, albuminuria, lymphocyte counts and waist to Hip ratio (Table 4). There was no significant association between gender, age, platelets, BMI, visceral fat, skeletal muscle fat, SBP, DBP, FPG, leptin, HDL-C, LDL, CRP, total cholesterol and evaluated markers of insulin resistance (serum Insulin and HOMA-IR score).

Table 1 Mean values of new and traditional cardiovascular risk factors

Variables	Mean ± SD	Range
Waist circumference (cm)	88.4±11.4	59–132
Hip circumference(cm)	100.8±13.3	61–138.5
Waist to Hip ratio	0.9±0.2	0.5–2.8
BMI(Kg/m^2)	27.5±8.5	16.7–45
Visceral fat(%)	7.1±8.2	1–54
Skeletal muscle fat(%)	26.7±6	11.3–43.8
Total body fat (%)	36.4±11.4	7.1–60
SBP (mmHg)	132.3±22.4	111–215
DBP (mmHg)	78.5±13	70–101
FPG(mmol/l)	5.1±0.09	4–9.3
Adiponectin(ng/ml)	67.4±55.3	4–224
Leptin	253.4±274.1	10.5–1063
Fasting Insulin(μU/l)	4.4±4.2	2–18.4
HOMA-IR Score	1.4±2.3	0.3–17.7
HDL-cholesterol (mmol/l)	1.3±0.5	0.1–3.9
LDL-cholesterol (mmol/l)	2.9±1	0.1–6.8
C-Peptide	1.2±0.8	0.2–4.5
CRP	3.1±27.5	1–224
Triglycerides (mmol/l)	1.8±1.5	0.5–12.8
Urea	4±1.8	1.7–10.7
Uric acid	2.4±1.1	1–6
Total cholesterol (mmol/l)	4.9±1.2	2.4–9.1
Albuminuria	221±253	1–1860.2

Discussion

The present study was designed to identify univariate and multivariate risk factors of variations in indicators of insulin resistance (increase in serum insulin or increase in HOMA-IR score) among black South Africans receiving first-line antiretroviral therapy.

Table 2 Univariate correlates of serum Insulin

Variable of interest	r coefficient	P value
Triglycerides	0.331	<0.0001
Serum Uric acid	0.437	0.002
Albuminuria	0.280	0.0001
Serum C-peptide	0.669	<0.0001
Lymphocytes	0.221	0.032
Waist to hip ratio	0.179	0.029

Table 3 Variables positively and significantly correlated to insulin
r esistance using HOMA-IR

Variable of interest	r coefficient	P value
Serum triglycerides	0.331	0.0001
Serum Uric acid	0.386	0.008
Albuminuria	0.316	<0.0001
Blood lymphocytes	0.186	0.04
Waist to hip ratio	0.217	0.013
Adiponectin	−0.322	0.035

The study sample was characterized by mean values of waist circumference, hip circumference, BMI and total body fat suggesting overweight/obesity, abdominal and peripheral obesity according to the recent cut off points of anthropometry specific for sub-Saharan Africa [24]. Studies from the current literature report that HIV–infected patients are increasingly overweight or obese at diagnosis and during antiretroviral therapy [24]. Obesity rates in both developed and developing countries such as South Africa are steadily becoming an epidemic [25–27]. Furthermore it is most probable that as HIV/AIDS patients live longer now than before advent of ARV's [3, 28] they will experience lower rates of AIDS-related wasting syndrome [29] due to the beneficial effects of antiretroviral therapy and consequently having higher rates of overweight and obesity [15, 30]. The predominance of women in this study may also explain the high levels of BMI, waist circumference and hip circumference as reported by several African studies [31, 32]. The demographics of the Eastern Cape Region of South Africa [33] where HIV positive population is more constituted by females [15] may also explain this discrepancy.

The present study showed a significant correlation between the majority of assessed cardiometabolic markers of atherosclerosis and indicators of insulin resistance such as serum insulin and HOMA-IR score. The significant and positive correlation demonstrated between serum triglycerides, serum uric acid, albuminuria, serum C-peptide, lymphocytes, waist to hip ratio and serum insulin suggest that these HIV/AIDS participants on antiretroviral therapy without protease inhibitors will in future be at a higher risk of cardiovascular disease such as coronary heart disease,

Table 4 Independent determinants of variations (adjusted R^2) of HOMA-IR score

Independent variables	Unstandardized β coefficient	Standard error	Standardized coefficient	P value
Constant	−0.701	0.286		0.019
Triglycerides	0.196	0.097	0.214	<0.049
C-Peptide	1.434	0.220	0.692	<0.0001

stroke, peripheral artery disease, chronic kidney disease and diabetes mellitus [4–11]. While the latter variables were also significantly and positively correlated with HOMA-IR score, there was a significant and negative correlation between serum adiponectin and HOMA-IR score. However there was no significant association of C-peptide with HOMA-IR score in these HIV/AIDS participants receiving the first-line antiretroviral therapy.

These findings suggest that several complex and complimentary mechanisms are involved in HIV/AIDS-related insulin resistance that leads to diabetes mellitus, chronic kidney disease and atherosclerosis. The following process may be considered. There could be atypical presentation of components of the metabolic syndrome in black African women. These may include infections, emerging role of adipokines (adiponectin, leptin) as mediators in atherosclerosis, exacerbation of chronic inflammatory state and innate immunity. Also included will be endothelial dysfunction, insulin resistance and dyslipidemia.

The present study showed a tendency to an increased level of serum insulin and HOMA-IR in participants not treated with protease inhibitors. They are mostly incriminated in HAART-related insulin resistance [34] and HIV–lipodystrophy syndrome. The present study also confirmed that adiponectin, but not leptin correlated with insulin sensitivity and a possible mechanism is reduced adiponectin mRNA in dystrophic fat [35, 36].

The multivariate analysis showed that after adjusting for gender, age, uric acid, adiponectin, lymphocytes and waist to hip ratio and in considering C-peptide, only triglycerides and C-peptide were identified as the independent determinants of high variance of HOMA-IR score in these black Africans exposed to antiretroviral therapy without protease inhibitors. The increase in triglyceride levels which was earlier observed before the antiretroviral therapy era was due to HIV infection itself [37]. HIV/AIDS is a multisystemic disease with potential for alteration of metabolic and endocrine functions. There is evidence that efavirenz increases triglyceride levels. Serum insulin, serum C-peptide and HOMA-IR measurements are significantly higher among HIV infected participants receiving protease inhibitors than in HIV seronegatives [38]. The present study is one of the few to report a significant and positive correlation between C-peptide and insulin resistance in HIV–infected participants not treated with protease inhibitors. The aetiology of insulin resistance seems to be multifactorial. In addition to the first-line antiretroviral therapy increase in triglyceride levels and C-peptide should be considered; in addition there is the HIV infection itself, including the virally encoded molecules Vpr and Tat (virion associated accessory proteins) which could contribute to the development of insulin resistance [39].

Clinical implications and public health perspectives

Insulin resistance is one of the components of metabolic syndrome. Other factors known to cause insulin resistance other than HIV infection such as obesity, physical inactivity, use of certain drugs like glucorticoids and niacin, and infections such as hepatitis should be controlled in HIV/AIDS participants receiving first-line antiretroviral therapy. The side effects of antiretroviral therapy may jeopardise the long term benefits of this treatment.

The study was limited to some degree because its cross sectional design was not able to demonstrate any causal association and because of our low resource setting we were unable to undertake the hyperinsulinemic euglycemic clamp studies.

In conclusion, there is a significant and univariate association between the majority of metabolic syndrome components, decrease in adiponectin, and insulin resistance in these black HIV/AIDS participants on antiretroviral therapy without protease inhibitors. C-peptide and triglycerides are the significant and independent determinants of 64.5 % variations of HOMA-IR score.

Conflict of interest None declared.

References

1. Hellerstein MK, Grunfeld, Wu K, Christiansen M, Shackleton CH. Increased de novo hepatic lipogenesis in human immunodeficiency virus infection. J Clin Endocrinol Meta. 1993;76:559–65.
2. Grunfeld C, Pang M, Jensen P, Feingold KF. Lipids, lipoproteins, triglycerides clearance, and cytokines in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. J Acquir Immune Defic Syndr. 1992;74:1045–52.
3. Mocroft A, Ledergerber B, Kathama C, Kirk O, Reiss, d'Arminio Monforte A, Knysz B, Dietrich M, Phillips AN, Lundgren JD, Euro-SIDA study Group. Decline in the AIDS and Death rates in the EuroSida study: an observational study. Lancet. 2003;362:22–9.
4. Aberg AJ. Cardiovascular complications in HIV Management: Past, Present, and Future. J Acquir Immune Defic Syndr. 2009;50:54–64.
5. Szczech LA, Grunfeld C, Scherzer R, Carlb C, Scherzer R, Canchola JA, Van der Horst C, Sidney S, Wohl D, Shlipak MG. Microalbuminuria in HIV infection. AIDS. 2007;21:1003–9.
6. Pao V, Lee GA, Grunfeld C. HIV therapy, metabolic syndrome, and cardiovascular risk. Curr Atheroscler Rep. 2008;10:61–70.
7. Grunfeld C. Insulin Resistance in HIV Infection. Top HIV Med. 2008;16:89–93.
8. Crane HM, Grunfeld C, Willig JH, Mugavero MJ, Van Rompaey S, Moore R, Rodriguez B, Feldman BJ, Lederman MM, Saag MS, Kitahata MM. Impact of NRT's on lipid level among a large HIV – infected cohort initiating antiretroviral therapy in clinical care. AIDS. 2011;25:185–95.
9. Feingold KR, Grunfeld C. The role of HDL in innate immunity. J Lipid Re. 2011;52:1–3.
10. Choi AI, Li Y, Deeks SG, Grunfeld C, Volberding PA, Schilpak MG. Association between kidney function and albuminuria with cardiovascular events in HIV –infected persons. Circulation. 2010;121:651–8.
11. Cahn P, Leite O, Rosales A, Cabello R, Alvarez CA, Seas Bolt C, L'Halien GP, Mantilla P, Derbis P, Zala C, Suffert T. Metabolic profile and cardiovascular risk factors among Latin American HIV infected patients receiving HAART. Braz J Infect Dis. 2010;14:158–66.
12. DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of NIDDM. A balanced overview. Diabetes Care. 1992;15:318–68.
13. Mathews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28:412–9.
14. Longo-Mbenza B, Mvindu HN, Kasiam On'Kin Jb, Nkakudulu B, Kianu B, Okwe N, Kabangu N. The deleterious effects of physical inactivity on elements of insulin resistance and metabolic syn- drome in Central Africans at high cardiovascular risk. Diab Met Syndr: Clin Res Rev. 2010.doi:10.1016/j.sx.2010.0555.001.
15. Awotedu K, Ekpebegh C, Longo-Mbeza B, Iputo J. Prevalence of metabolic assessed by IDF and NCEP ATP 111 criteria and determinants of insulin resistance among HIV patients in the Eastern Cape Province of South Africa. Diab Met Syndr: Clin Res Rev. 2010;4:210–4.
16. World Medical Association; Ethical principles for medical research involving human subjects. 59th WMA General Assembly, Seoul, October 2008. http://www.wma.net.
17. Baskon M, Os I, Sandvik L, Oektedalen O. Microalbuminuria associated with indicators of inflammatory activity in an HIV- positive population. Nephrol Dial Transplant. 2008;23:3130–7.
18. Stehouwer CD, Nauta JJ, Zeldenrust GC, Hackeng WH, Doncker AJ, den Oholander GJ. Urinary albumin excretion, Cardiovascular disease and endothelial dysfunction in non-insulin-dependent diabetes mellitus. Lancet. 1992;340:319–23.
19. Russo LM, Comper WD, Osieka TM. Mechanism of albuminuria associated with cardiovascular disease and kidney disease. Kidney Int-Suppl. 2004;66:S67–8.
20. Walcher D, Marx N. C-Peptide in the vessel wall. The Review of Diabetes Studies. 2009;6:180–6.
21. Longo-Mbenza B, Luila EL, Mbete P, Vita EK. Is hyperuricemia a risk factor of stroke and coronary heart disease among Africans? Int J Cardiol. 1999;71:17–22.
22. Duncan BB, Schmidt MI. Chronic activation of the innate immune system may underlie the metabolic syndrome. Sao Paulo Med J. 2001;119:12–27.
23. Hanson GK, Libby P, Schonbach U, Yan Z-Q. Innate and Adaptive Immunity in the pathogenesis of Atherosclerosis. Circ Res. 2002;91:281–91.
24. Kasiam Lasi On'kin JB, Longo-Mbenza B, Nge Okwe A. Survey of abdominal obesities in an adult urban population of Kinshasa Democratic Republic of Congo. Cardiovascular J Afr. 2007;18:300–7.
25. Micciolo R, Di Francesco V, Fantin F, Canal L, Harris TB, Bosello O, Zamboni M. Prevalence of overweight and obesity in Italy (2001–2008): is there a rising obesity epidemic? Ann Epidemiol. 2010;20:258–64.
26. Olatunbosun ST, Kaufman JS, Bella AF. Prevalence of obesity and overweight in urban adult Nigerians. Obes Rev. 2010, 6. doi:10.1111/j.1467-789X.2010.00801.x.
27. Tuei VC, Maiyoh GK, Ha CE. Diabetes mellitus and obesity in sub-Saharan Africa. Diabetes Metab Res Rev. 2010;26:433–45.
28. Antiretroviral therapy cohort collaboration life expectancy of individual s on combination antiretroviral therapy in high income countries: a collaborative analysis of 14 cohort studies. Lancet. 2008;372:293–9
29. Hodgson LM, Ghattas H, Prichitt H, Schwenk A, Payne L, Macallan DC. Wasting and obesity in HIV outpatients. AIDS. 2001;15:2341–2.

30.
Cianflone NC, Roediger MP, Eberly L, Headd M, Marconi V, Ganesan A, Weintrob A, Barthel RV, Fraser S, Agan BK. Infec- tious Disease Clinical Research Program HIv working Group. Increasing rates of obesity among HIV infected persons during the HIV epidemic PLoS one. 2010;5:e10106.
31. Jarvie JL, Johnson CE, Wan Y, Aslam F, Athanasopoulos
LV, Pollin I, Foody JM. Geographic variance of cardiovas- cular risk factors among community women: the national sister to sister campaign. J Womens Health (Larchmt).
2011;20:11
–9.
32. Kahn SK. Can racial disparity in health between black and white Americans be attributed to racial disparities in body weight and socioeconomic status? Health Soc Work. 2010;35:257–66.
33. WWW.mrc.ac.za/bod/easterncape. hdg c accessed on 13th August
20
10.
34. Walli R, Herfort O, Michel GM, Demant T, Jager H, Dieterle C, Bogner JR, Landgraf, Goebel F. Treatment with protease inhibi- tor4s associated with peripheral insulin resistance and impaired
oral glucose tolerance in HIV −1 infected patients. AIDS.
1998;12: F51–8.
35. Komski L, Kuritzkes D, Lichtenstein K, Eckel R. Adipocyte de- rived hormone levels in HIV lipodystrophy. Antiviral Ther.
2003;8:9–15.
36. Tong O, Sankale J, Hadigan C, Tan G, Rosenberg E, Kanki P, Grinspoon S, Hotamisligil G. Regulation of Adiponectin in human immunodefi- ciency virus –infected patients: relationship to body composition and metabolic indices. J Clin Endocrinology Metab. 2003;88:1559–64.
37. Isezuo SA, Makusidi MA. Metabolic dysfunction in non – antiretro-
viral treated HIV/AIDS patients. Niger J Clin Pract. 2009;12:375–8.
38. Rondanelli M, Caseli D, Trotti R, Solerte SB, Maghnie M, Maccabruni A, Minoli L, Ferrari E. Endocrine Pancreatic Dysfunction in HIV – Infected children: Association with Growth Alterations. JID.
2004;190:908–12.
39. Kino T, Mirani M, Alesi S, Chrousos GP. AIDS –related lipodys- trophy/insulin resistance syndrome. Horm Metab Res. 2003;35:129–
36.

ORIGINAL ARTICLE

Interaction between essential trace and toxic elements in the scalp hair samples of smokers and alcohol user diabetics

Hassan Imran Afridi & Tasneem Gul Kazi & Dermot Brabazon & Sumsun Naher

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Abstract In the present study, trace and toxic elements were determined in the Scalp Hair (SH) samples of patients diagnosed with diabetes mellitus (DM) who were smokers and habitual alcohol drinkers living in Dublin, Ireland. The concentrations of elements were measured by inductively coupled plasma atomic emission spectrophotometer after microwave-assisted acid digestion. The validity and accuracy of the methodology was checked using Certified Reference Material (CRM) (NCS ZC 81002b) and by the conventional wet acid digestion method on the same CRM. The results of this study showed that the mean values of cadmium, copper, iron, nickel and lead were significantly higher ($P < 0.001$), in scalp hair samples of diabetic patients as compared to referents of both gender. While the smokers and alcohol drinker referents and DM patients have two to three time higher values of these elements than those subjects who were not smokers and teetotallers. The concentrations of zinc, chromium and manganese were lower in the scalp hair samples of diabetic patients as compared to referents. The deficiency of zinc, chromium and manganese, while the high exposure of cadmium, lead and nickel, as a result of cigarette smoking and alcohol consumption, may be synergistic with risk factors associated with diabetes.

Keywords Diabetes mellitus · Scalp hair · Cigarette smokers · Alcohol drinking · Trace and toxic elements · Inductively coupled plasma atomic emission spectrophotometer

H. I. Afridi (✉)
e-mail: hassanimranafridi@yahoo.com

Introduction

Clinical research suggests that the body's balance of trace elements and minerals can be disrupted by diabetes mellitus [1, 2]. Conversely, research also suggests that early imbalances of specific elements may play an important role in upsetting healthy glucose metabolism and insulin action. With regard to essential trace elements, the main clinical interest and the majority of publications focus on deficiencies of a single element or a combination of elements. Trace element deficiencies mostly occur in combination with chronic diseases and malabsorption. Chronic hyperglycemia can cause significant alterations in the status of some micronutrients and furthermore, some of these nutrients can directly modulate glucose homeostasis [3, 4]. The deficiencies of certain minerals such as magnesium (Mg), zinc (Zn) and chromium (Cr) have been shown to predispose a person to glucose intolerance and to promote the development of diabetic complications [5].

It was reported that Zn is involved in the synthesis, storage, secretion and conformational integrity of insulin, Zn and insulin monomers assemble to a dimeric form for storage and secretion as crystalline insulin [6]. Lower level of Zn in body may affect the ability of the islet cells of the pancreas to produce and secrete insulin, particularly in type-2 diabetes [7]. Many epidemiological studies reported the decreased plasma Zn and intracellular Zn concentrations, and increased urinary Zn excretion compared to non- diabetic subjects. The metabolic disorder type 2 diabetes

mellitus increases the risk of coronary heart disease by a factor of two to four times and is a major cause of mortality among diabetic patients [8–10]. It was intensively investigated that chromium (Cr) acts as a powerful blood glucose modulator that can help guard against glucose imbalances [11]. It tends to lower glucose response in individuals with elevated levels and heighten glucose response in those with insufficient levels. Insufficient dietary Cr intake has also been implicated as a possible risk factor for the development of diabetes [12].

The metabolisms of other micronutrients such as copper (Cu), iron (Fe) and manganese (Mn) have been reported to be altered in diabetes. Manganese is a cofactor for a number of enzymatic systems including arginase which has been found to be elevated in diabetic rats [13]. Mn levels are required for development of normal insulin synthesis and secretion [14, 15]. Diabetes mellitus is associated with altered iron homeostasis in both human and animal diabetic models. Iron is a metal oxidant, capable of generating reactive oxygen species and has been postulated to contribute to diabetic nephropathy. Excess iron has been implicated in the pathogenesis of diabetes and its complications [16, 17].

Cigarette manufacturing design has evolved considerably over the last few decades with the incorporation of new tobacco processes, papers, filters and several ingredients (flavour, humectants and casing materials), which either alone or in combination have the potential to modify the quantity and/or the quality of the smoke yielded [18]. The tobacco plant absorbs toxic elements most probably from the soil, fertilizers or from pesticides [19, 20]. Other environmental factors that may influence the uptake of toxic elements by tobacco plants include the pH of soil, contaminated irrigated water and sewage sludge used as fertilizers. Smoking delivers heavy metals (the term is used for some lighter metals and metalloids) to the lungs [18], particularly the more volatile metals such as cadmium (Cd) and mercury that partition preferentially into the smoke phase on combustion [21]. Some of these readily pass into the bloodstream and may accumulate in specific organs [22]. Indeed smoking has long been considered a major source of several heavy metals in blood and various organs, and Cd in particular is regarded as one of the “strong carcinogens” in tobacco smoke [23] with nickel (Ni) and arsenic (As) are currently classified “carcinogenic to humans” by the International Agency for Research on Cancer (IARC) among 87 mainly organic carcinogens. Intake of alcohol is a generally accepted behavior, but it has a significant impact on health. Cross sectional investigations have demonstrated a high prevalence of diabetes mellitus in drinkers, suggesting a possible contributory role for alcohol intake in the development of diabetes.

From some cross-sectional population studies, it was reported that diabetes could augment the risk of Cd induced renal damage, especially tubular dysfunction [24]. It was consistent with a previous study that diabetic patients may be more susceptible to the toxic effect of Cd on the renal proximal tubule [25]. Several experimental studies have demonstrated an increased susceptibility toward Cd nephrotoxicity [26] in spontaneously diabetic mice and hamsters, when compared with normal animals of the same strain. Streptozotocin-induced diabetic rats are more susceptible to Cd nephrotoxicity than are normal rats when they are exposed subchronically to Cd chloride in drinking water [27].

Determinations of trace elements in human tissues and fluids were used to obtain information on nutritional status for diagnosis of diseases, indication of systemic intoxication, and to obtain information on environmental exposure. In the majority of cases, whole blood, serum, plasma, and urine were analyzed [28]. Hair can provide a more permanent record of trace and toxic elements (TEs) associated with normal and abnormal metabolism as well as TEs assimilated from the environment. In addition, hair is easily collected, conveniently stored, and easily treated. Therefore, the analysis of human hair has become an important way to understand any quantitative change in certain elements inside the body [29]. One of the most widely used analytical technique for different elements determination in biological and environmental materials is inductively coupled plasma atomic emission spectrometry (ICP-AES) due to its advantages over other analytical methods; i.e. the possibility of simultaneous determination of many elements of interest, freedom from different chemical interferences and high detection power. ICP-AES also offers rapid, multi-element determinations. The sensitivity of ICP-AES is lower than that of either inductive coupled plasma mass spectrophotometer (ICP-MS) or atomic absorption graphite tube atomizer (AA-GTA), but ICP-AES can handle higher levels of total dissolved solids (TDS) than ICPMS and is much faster than AA-GTA. Since ICP-AES is able to analyze samples with higher TDS, more concentrated solutions can be prepared allowing trace elements to be measured. The main advantage of microwave-assisted samples pre-treatment is its requirement of small amount of mineral acids and a reduction in the production of nitrous vapors. Microwave systems keep blank levels low because only small volumes of reagents are required and allow more samples to be processed per hour than conventional digestion systems [30].

The aim and objective of our present study was to assess the concentrations of Cd, Cu, Cr, Fe, Mn, Ni, Pb and Zn in the scalp hair samples of smoker and alcohol user diabetic patients. For a comparative study, 88 non- diabetic individuals (smoker and alcohol user) of the same age group (ranged 35–55 years), belongs to same socioeconomic status, localities and dietary habits were selected as referents.

The elements under study were analyzed by inductively coupled plasma atomic emission spectrophotometer, prior to microwave-assisted acid digestion.

Materials and methods

Apparatus

A Varian Liberty 220 (Mulgrave, Victoria, Australia) Inductively Coupled Plasma Atomic Emission Spectrometer with the axially viewed plasma was used for the analysis. The Liberty Series II ICP features a 40 MHz free running RF generator, a 0.75 m Czerny-Turner monochromator with 1,800 grooves/mm holographic grating used in up to four orders. The resolution of the spectrometer is typically 0.018 nm in 1st order, 0.009 nm in 2nd order, 0.007 nm in 3rd order and 0.006 nm in 4th order. The instrument was controlled with a Digital Equipment Corporation (DEC) Venturis computer with an Intel Pentium processor and Varian Plasma 96 software running under Microsoft Windows 95 operating system. The instrumental conditions are shown in Tables 1 and 2. A Hinari Life style (Elstree, Hertfordshire, England) domestic microwave oven (maximum heating power of 800 W) was used for digestion of the scalp hair samples. Acid-washed polytetrafluoroethylene (PTFE) vessels and flasks were used for preparing and storing solutions.

Reagents and glass wares

Ultrapure water obtained from ELGA Lab Water system (Bucks, UK) was used throughout the work. Concentrated nitric acid (65 %) and hydrogen peroxide (30 %) were obtained from Merck (Darmstadt, Germany), and checked for possible trace metal contamination. Working standard solutions of Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn were prepared immediately prior to their use, by stepwise dilution of certified standard solutions (1,000 ppm) Fluka Kamica (Buchs, Switzerland), with 0.5 M HNO_3. All solutions were stored in polyethylene bottles at 4 °C. For the accuracy of methodology, the certified reference material (CRM), human hair NCSZN 81002b (Beijing, China), was used (Table 3). All glassware and plastic materials used were previously soaked for 24 h in 5 M nitric acid, washed with distilled and finally rinsed with ultrapure water, dried, and stored in a class 100 laminar flow hoods.

Sample collection and pretreatment

An epidemiological cross-sectional survey was conducted among 150 referents and 129 DM subjects of both genders, age ranged 35–55 years, living in urban area of Dublin,

Table 1 Measurement conditions for inductively coupled plasma atomic emission spectroscopy

Parameters	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Wavelength (nm)	226.502	267.716	327.396	238.204	259.373	231.604	220.553	213.8
Height (mm)	3	5	5	5	5	5	3	5
Windows (nm) (above the coil)	0.027	0.040	0.027	0.027	0.027	0.027	0.027	0.027
Scan (nm)	0.040	0.060	0.040	0.040	0.040	0.040	0.040	0.040
Integration (s)	3	3	3	3	3	3	3	3
Replicates	3	3	3	3	3	3	3	3
Sample uptake (sec)	30	30	30	30	30	30	30	30
PMT (V)	650	650	650	650	650	650	650	650
Power (kW)	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10
Plasma flow (L/min)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Auxiliary flow (L/min)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Pump speed (rpm)	15	15	15	15	15	15	15	15
Background mode	Dynamic	Dynamic	Dynamic	Dynamic	Dynamic	Dynamic	Dynamic	Dynamic
Max curve order	1	1	1	1	1	1	1	1
CC limit	0.995	0.995	0.995	0.995	0.995	0.995	0.995	0.995

Table 2 Liberty 220 common parameters

Nebulizer type	V-groove
Nebulizer pressure	150 kPa
Stabilization time	10 s
Sample delay time	30 s
Rinse time	10 s
Pump- tube	Orange- orange (inlet)
	Blue- blue (outlet)
Snout purge	off
Fast pump	On

Ireland (Table 4). Before the start of this study, all referents and diabetic patients of both genders, age range 35–55 years, were informed through a consent form by the administration about the aim of study, and all agreed to participate and signed the form. A questionnaire was also administered to them to collect details regarding physical data, ethnic origin, health, duration of smoking and drinking the alcohol, frequency of smoking and drinking alcohol, dietary habits, age, and consent. The criteria for the diagnosis of diabetes mellitus by a positive glucose tolerance test showing fasting blood glucose >140 mg/dl (>7.7 mmol/L) and postprandial blood glucose >200 mg/dl (>11.1 mmol/L) 2 h after 75 g of oral glucose. The patients were grouped according to their habits, non-smoking or non-alcohol users patients (PNACS), cigarette smoking patients (PCS), alcohol users patients (PAD), and patients who had both habits (PACS). While control group are also divided into four groups: non-smoking or non-alcohol users referents (RNACS), cigarette smoker referents (RCS), alcohol users referents (RAD) while last group had alcohol consumption with cigarette smoking (RACS) as shown in Table 4.

Physical examinations were carried out in a basic health unit of Dublin, Ireland to measure participant's weight, height, blood pressure and biochemical data. For all patients and referents, anthropometric parameters including weight, height and waist circumference were measured using the standard protocols (Table 5). There were no statistically significant differences between both groups of patients and referents with regard to height and weight. The study protocol was approved by the local ethics committee of Dublin city university, Ireland.

In diabetic patients, the duration of diabetes was 8–12 years. 23 % of patients in our survey had documented vascular disease (9 % having a history of cardiovascular disease, 12 % had hypertension and were receiving antihypertensive therapy). 50 % of the patients in this survey were obese while 50 % of patients also received insulin. The criteria of healthy subjects included no history of symptoms of diabetes and any coronary disease, documented in their medical notes. All control subjects underwent a routine medical examination. All patients and controls/referents were requested to complete an interviewer-administered questionnaire, concerning their demographic characteristics, age, health history, lifestyle habits, and diet. They gave written consent to participate in the study.

Collection of scalp hair samples

The hair samples (~1.0 g each) were taken from the nape of the neck. Hair samples were put into separate plastic envelopes for each participant, on which the identification (ID) number of the participant was indicated. The plastic envelope of each subject was tightly sealed and attached to a questionnaire. Before analysis, each individual hair sample was cut into approximately 0.5 cm long pieces and mixed to allow a representative sub-sampling of the hair specimen. After cutting, each sample was washed with diluted Triton X-100: samples were then rinsed with distilled water and then with deionized water. The samples were then rinsed three times with acetone [28]. The samples were then dried in an oven at 75±5 °C for 2 h. Dried samples were stored separately in polyethylene bags.

Table 3 Determination of trace elements in certified sample of human hair (CRM) by conventional (CDM) and microwave digestion method (MWD) (n0 10) (µg/g)

Elements	Conventional digestion method CDM µg/g	Microwave digestion method MWD µg/g	T value^	% recovery$	Certified values µg/g
Cd	0.0716±0.003 (4.19)	0.0714±0.006 (8.40)	0.305	99.7	0.072±0.010
Cr	8.72±0.73 (8.37)	8.67±0.49 (5.65)	0.902	99.4	8.74±0.97
Cu	33.5±1.92 (5.73)	33.0±1.58 (4.79)	0.193	98.5	33.6±2.3
Fe	158±6.73 (4.26)	154±8.72 (5.66)	0.182	97.5	160±16
Mn	3.79±0.34 (8.97)	3.76±0.28 (7.45)	0.727	99.2	3.83±0.39
Ni	5.71±0.51 (8.93)	5.67±0.43 (7.58)	0.339	99.4	5.77*
Pb	3.80±0.37 (9.74)	3.72±0.35(9.41)	0.081	98.05	3.83±0.18
Zn	191±7.28 (3.81)	187±9.53 (5.09)	0.648	97.9	191±16

Key: ^Paired t-test between CDM and MWD, DF0 9, T (critical) at 95 % CI02.262, P0 0.05, * means in percentage, Values in ( ) are RSD

$ % recovery was calculated according to: $\frac{\text{MDM}}{\text{CDM}}$ 100

Table 4 Characteristics of study subjects (35–55) age groups

Parameters	Referents		Diabetic mellitus patients	
	Male (N089)	Female (N061)	Male (N070)	Female (N059)
Occupation				
Labour	38	24	32	24
Office workers	35	20	25	19
Not working	16	17	13	16
Habits				
Smoking tobacco	19 (21.4 %)	13 (21.3 %)	15 (21.4 %)	13 (22.0 %)
Alcohol drinkers	18 (20.2 %)	10 (16.4 %)	11 (15.7 %)	15 (25.4 %)
Smoking tobacco +Alcohol drinkers	16 (18.0 %)	12 (19.7 %)	13 (18.6 %)	13 (22.0 %)
Non smoking tobacco and alcohol drinkers	36 (40.4 %)	26 (42.6 %)	31 (44.3 %)	18 (30.5 %)

Microwave-assisted acid digestion

A microwave-assisted digestion (MWD) procedure was carried out, in order to achieve a shorter digestion time. Duplicate samples of scalp hair (200 mg) of each diabetic patients and control individuals were directly placed into Teflon PFA flasks. Two milliliters of a freshly prepared mixture of concentrated HNO_3–H_2O_2 (2:1, v/v) were added to each flask and kept for 10 min at room temperature then placed in a covered PTFE container. This was then heated follow- ing a one-stage digestion program at 80 % of total power (800 W). Complete digestion of scalp hair samples required
5–8 min. After the digestion, the flasks were left to cool and the resulting solution was evaporated to semi-dried mass to remove excess acid. About 5 ml of 0.1 M nitric acid was added to the residue and filtered through a Whatman no. 42 filter paper and diluted with deionized water up to 10.0 ml in volumetric flasks. Blank extractions were carried through the complete procedure. Blanks and standard solutions were prepared in a similar acid matrix. The validity and efficiency of the MWD method was checked with certified values of human hair NCSZC 81002b and with those obtained from conventional wet acid digestion method [29, 30].

Analytical figures of merit

Data processing and statistical analysis were conducted by using the computer program EXCEL (XP 2002; Microsoft Corp., Redmond, WA) and Minitab 13.2 Minitab Inc., (State College, PA) software packages. Normally distributed data were expressed as mean ± SD, Student's t-test and Mann–Whitney test were used to assess the significance of the differences in concentrations of elements among study subjects. All tests were two-sided and a P value of <0.05 was considered significant.

Calibration was performed with a series of Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn standards. Sensitivity (m) was the slope value obtained by least-square regression analysis of calibration curves based on absorbance signals. The equation (N05) for the calibration curves was as follows:

Y ¼ ð1:28 10 3 8:60 10 5ÞðCdÞ Þ ð1:30 10 3 1:23 10 4Þ; r ¼ 0:999
Y ¼ ð1:198 10 3 5:7 10 5ÞðCrÞ Þ ð1:167 10 3 4:59 10 4Þ; r ¼ 0:999
Y ¼ ð4:38 10 2 7:11 10 3ÞðCuÞ Þ ð4:39 10 2 7:25 10 3Þ; r ¼ 0:999
Y ¼ ð1:38 10 3 9:41 10 4ÞðFeÞ Þ ð1:40 10 3 8:07 10 4Þ; r ¼ 0:999
Y ¼ ð7:12 10 3 3:1 10 5ÞðMnÞ Þ ð6:98 10 3 2:91 10 5Þ; r ¼ 0:999
Y ¼ ð1:66 10 2 2:24 10 3ÞðNiÞ Þ ð1:73 10 2 2:92 10 3Þ; r ¼ 0:999
Y ¼ ð1:875 10 2 7:40 10 4ÞðPbÞ Þ ð1:91 10 2 5:83 10 3Þ; r ¼ 0:999
Y ¼ ð7:83 10 2 1:18 10 2ÞðZnÞ Þ ð8:51 10 2 1:03 10 2Þ; r ¼ 0:999

where Y is the integrated absorbance, r is the regression and the concentration range of Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn for calibration curve reached from the detection limits up to 500 μg/L. The limit of detection, equal to 0.0003 ng/mg, 0.0004 ng/mg, 0.01 ng/mg, 0.01 ng/mg, 0.01 ng/mg, 0.01 ng/mg, 0.0003 ng/mg and 0.01 ng/mg for Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn respectively. 3 σ/m 'σ' being the standard deviation corresponding to ten blank injections and 'm' the slope of the calibration graph. The quantification limits, defined as 10 σ/m were calculated as: 0.0009 ng/mg, 0.0013 ng/mg, 0.03 ng/mg,
0.03 ng/mg, 0.03 ng/mg, 0.05 ng/mg, 0.001 ng/mg and 0.04 ng/mg for Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn respectively.

Table 5 Clinical and biochemical characteristics of diabetic patients and referents

	Referents				Patients			
	RNACS	RACS	RCS	RAD	PNACS	PACS	PCS	PAD
Male								
Height (cm)	179.2±1.34	180±0.54	179.3±1.2	177.3±1.25	180.2±1.44	179.5±1.24	178.3±1.25	182.0±1.24
Weight (kg)	78.7±1.25	82.4±1.53	81.8±1.06	82.6±1.48	80.7±1.36	82.9±1.76	83.3±1.47	83.8±1.73
Waist circumference (cm)	75.9±1.25	79.5±1.34	78.7±1.23	82.7±1.27	85.9±1.45	81.3±1.17	86.2±1.35	87.9±1.52
BMI (kg/m^2)	24.5±1.59	25.4±1.28	25.4±1.39	26.3±1.42	24.8±1.28	25.7±1.86	26.2±1.15	25.3±1.47
Systolic BP (mmHg)	119.8±2.46	125.9±1.3	124.3±1.9	126.9±1.31	120.9±1.57	128.2±1.69	124.5±1.22	129.9±1.39
Diastolic BP (mmHg)	79.6±2.3	79.4±1.28	80.3±1.42	81.2±1.53	85.4±1.05	88.2±1.46	83.9±1.37	86.5±1.16
Fasting plasma glucose (mmol/l)	(90, 99)	(95, 106)	(92, 100)	(93, 102)	(131, 187)	(149, 213)	(143, 192)	(145, 202)
Fasting plasma insulin (mmol/l)	4.29±0.13	4.87±0.24	4.42±0.45	4.75±0.09	6.65±0.24	8.34±0.22	7.49±0.63	7.87±0.48
Diabetes duration (year)	–	–	–	–	9.2±0.32	11.5±0.64	10.6±0.57	11.8±1.28
Female								
Height (cm)	164.0±1.03	163.7±0.7	162.9±1.2	163.8±1.35	164.8±1.52	162.9±0.81	163.7±1.24	163.1±0.79
Weight (kg)	60.4±1.13	63.6±1.27	62.8±0.54	63.9±1.08	62.5±1.36	64.7±1.52	63.9±1.72	65.3±1.12
Waist circumference (cm)	63.1±0.51	63.6±1.34	65.3±1.17	64.6±1.32	63.7±1.52	64.9±0.65	64.3±0.76	65.3±0.63
BMI (kg/m^2)	22.5±1.32	23.7±1.18	23.7±1.31	23.8±1.69	23.0±0.62	24.4±1.35	23.8±1.09	24.5±1.35
Systolic BP (mmHg)	119.6±1.09	120.2±1.2	119±0.76	120.6±1.18	120.2±1.36	121.7±1.16	122.4±1.03	124.5±1.26
Diastolic BP (mmHg)	79.9±1.42	81.3±1.05	81.8±0.73	82.1±0.83	80.3±1.16	82.5±1.08	82.9±1.25	84.3±1.05
Fasting plasma glucose (mmol/l)	(85, 99)	(94, 103)	(92, 100)	(93, 105)	(135, 192)	(146, 202)	(140, 195)	(141, 198)
Fasting plasma insulin (mmol/l)	4.36±0.25	4.85±0.16	4.40±0.37	4.68±0.22	6.53±0.47	8.52±0.19	7.47±0.54	7.93±0.42
Diabetes duration (year)	–	–	–	–	10.4±0.59	10.6±0.73	11.4±1.24	10.5±1.72

BMI body mass index, RNACS non-smoking or non-alcohol users referents, RACS referent who were cigarette smokers and alcohol users, RCS referent cigarette smokers, RAD referent alcohol drinkers, PNACS non-smoking or non-alcohol users patients, PACS patient who were cigarette smokers and alcohol users, PCS patient cigarette smokers, PAD patient alcohol drinkers

Result

In the study population, ~26 % DM patients and ~35 % referents of both genders were smokers, whilst 24–28 % DM patients and 24–34 % referents of both genders were alcohol drinkers. The physical parameters of both groups of patients and referents were obtained by a standard method as shown in Table 5. The weight, body mass index, and blood pressure (systolic and diastolic blood pressure) levels of DM patients were higher than those in healthy referents, but there is no significant difference ($P < 0.05$). The smoker and alcohol drinker referents weighed more than nonsmoker referents (P00.042).

The elemental contents in the scalp hair samples varied widely among individuals; thus, a significantly large number of samples were required for statistical interpretation of the data to achieve a meaningful correlation between physiological disorders and concentrations of trace and TEs. The mean concentrations with standard deviations of each element in scalp hair samples shown in Table 6, indicate that the concentrations of the essential trace element (Cr, Mn and Zn) were lower in the scalp hair samples of all groups of DM patients, whilst the level of Cd, Cu, Fe, Ni and Pb were elevated (not significantly) in the scalp hair samples of DM patients, but significant difference was found in smoker and alcohol drinker DM patients with respects to the referents. In the case of essential trace elements, Cr, Mn, Zn, no significant difference was observed for RCS with respect to the RNACS, although the levels of toxic elements were higher in their scalp hair samples.

The concentrations of Zn in the scalp hair samples of male RNACS and RCS were significantly higher at 95 % (CI: 3.58, 4.25) and (CI:3.46, 3.82) μg/g, respectively, compared with those in PNACS and PCS, (CI: 152, 161) and (CI: 131, 140) μg/g, respectively, with $P < 0.001$. The Zn levels in the scalp hair samples of RACS and RAD, (CI: 155, 169) and (CI: 136, 145) μg/g, respectively, were found to be higher than those in PACS and PAD, (CI: 99.7, 112) and (CI: 112, 125) μg/g, respectively, (P00.001–0.002). The same trend was observed in female patients and referents (Table 6).

The concentrations of Cr in the scalp hair samples of female RNACS and RCS were significantly higher at 95 % (CI: 3.82, 4.04) and (CI: 2.51, 2.83) μg/g, respectively, compared with those in PNACS and PCS, (CI: 2.54, 2.77) and (CI: 2.17, 2.34) μg/g, respectively, with $P < 0.001$. The Cr levels in the scalp hair samples of RACS and RAD, (CI: 2.95, 3.13) and (CI: 3.27, 3.42) μg/g, respectively, were found to be higher than those in PACS and PAD, (CI: 1.84,

Table 6 Concentrations of trace and toxic metals in scalp hair samples of referent and Diabetic mellitus subjects (μg/g)

Elements	Referents				Patients			
	RNACS	RACS	RCS	RAD	PNACS	PACS	PCS	PAD
Male								
Cadmium	0.68±0.15*	1.95±0.15	1.52±0.23	1.73±0.22	2.26±0.32	3.79±0.39	2.93±0.32	3.48±0.46
Copper	11.6±1.25	14.7±1.37	12.9±1.06	13.5±1.63	14.7±1.05	17.8±1.31	15.2±1.13	16.6±1.37
Iron	19.6±2.51	26.9±1.74	23.9±2.32	25.7±1.05	22.3±1.37	28.1±1.49	24.7±2.31	27.3±1.69
Nickel	3.42±0.36	4.74±0.69	3.56±0.93	4.53±0.79	4.95±0.67	7.83±1.25	6.34±0.62	7.54±0.52
Lead	3.63±0.37	5.98±0.43	4.51±0.18	5.48±0.36	6.42±0.50	9.43±1.12	7.62±0.28	8.79±0.45
Chromium	3.86±0.18	3.10±0.07	3.52±0.24	3.33±0.14	2.58±0.14	1.92±0.17	2.30±0.20	2.12±0.08
Manganese	3.93±0.21	2.14±0.12	2.67±0.31	2.36±0.23	2.10±0.22	1.27±0.24	1.65±0.19	1.36±0.16
Zinc	252±13.6	164±14.8	186±9.5	141±7.52	157±9.3	105±12.4	136±11.5	118±12.9
Female								
Cadmium	0.62±0.07	2.04±0.16	1.56±0.12	1.87±0.16	1.76±0.06	3.63±0.19	2.72±0.15	3.05±0.32
Copper	10.7±1.03	17.9±1.07	16.5±1.28	16.8±1.31	17.3±0.85	25.7±1.06	23.2±1.01	26.1±0.46
Iron	19.9±1.64	25.7±0.57	23.3±0.92	24.7±0.97	25.2±0.83	30.7±3.28	27.4±1.36	29.2±0.53
Nickel	3.35±0.47	5.85±0.31	5.32±0.24	5.58±0.29	4.42±0.36	7.63±1.15	6.51±0.52	7.28±1.31
Lead	3.52±0.21	5.67±0.38	4.35±0.47	5.49±0.36	6.36±0.47	9.17±1.37	7.48±0.27	8.56±0.63
Chromium	3.94±0.33	3.04±0.16	3.65±0.35	3.42±0.28	2.65±0.21	1.49±0.25	2.26±0.18	2.03±0.19
Manganese	4.35±0.15	3.26±0.26	3.94±0.29	3.58±0.17	2.35±0.23	1.52±0.44	1.78±0.16	1.49±0.26
Zinc	245±10.5	143±7.52	197±9.32	152±8.56	148±12.9	54.3±6.42	115±7.92	65.7±7.12

Key: * Mean ± standard deviation, RNACS non-smoking or non-alcohol users referents, RACS referent who were cigarette smokers and alcohol users, RCS referent cigarette smokers, RAD referent alcohol drinkers, PNACS non-smoking or non-alcohol users patients, PACS patient who were cigarette smokers and alcohol users, PCS patient cigarette smokers, PAD patient alcohol drinkers

2.02) and (CI: 2.05, 2.16) μg/g, respectively, (P < 0.001). The same trend was observed in male patients and referents (Table 6).

The concentrations of Mn in the scalp hair samples of male RNACS and RCS were significantly higher at 95 % confidence interval (CI) (3.82, 4.04) and (2.51, 2.83) μg/g, respectively, compared with those in PNACS and PCS, (CI: 1.99, 2.21) and (CI: 1.56, 1.75) μg/g, respectively, with P < 0.001. The Mn levels in the scalp hair samples of RACS and RAD, (CI: 2.07, 2.19) and (CI: 2.24, 2.47) μg/g, respectively, were found to be higher than those in PACS and PAD, (CI: 1.15, 1.40) and (CI: 1.27, 1.43) μg/g, respectively, (P < 0.001). The same trend was observed in female patients and referents (Table 6). It was observed that the level of Cr, Mn and Zn did not vary significantly in the scalp samples of referent smokers and alcohol users, indicating that the alteration of these trace metals in scalp hair samples of smokers and alcohol drinker diabetic patients was mainly because of the disease state of the patients.

The levels of Cu and Fe in scalp hair samples were statistically significantly higher (P < 0.01) in all groups of diabetic patients (PCS, PAD, PACS) compared with referent groups of both genders (Table 6). An elevated level of Cd content was observed in the scalp hair of smoker patients of both genders (P < 0.001) (Table 6). The ranges of Cd in the scalp hair samples of female RACS and RAD were (CI: 1.87, 2.03) and (CI: 1.62, 1.84) μg/g, respectively, whereas those in PACS and PAD were (CI: 3.61–3.97) μg/g and (CI: 3.25, 3.71) μg/g, respectively, (P < 0.002). The same trend was observed in male patients and referents (P < 0.001) (Table 6).

The Pb concentration in the scalp hair samples of male RNACS was 3.45-3.81 μg/g, (95 % CI), whereas in the PNACS, the Pb level was (6.17, 5.26) μg/g (Table 6). Similarly, a higher level of Pb was observed in PACS (CI: 8.92, 9.87) μg/g, PCS (7.46, 7.77) μg/g and PAD (CI: 8.56, 9.03) μg/g than in RNACS (P < 0.001). The same trend was observed in females (Table 6). The levels of Ni in the scalp hair samples of female RNACS and RCS were found to be lower, (3.25, 3.59) and (CI: 5.18, 5.47) μg/g, respectively, compared with those in PNACS and PCS, (4.45, 4.80) μg/g and (6.34, 6.90) μg/g, respectively. The concentration of Ni in scalp hair samples of RACS and RAD were (5.68, 6.03) and (5.43, 5.74) μg/g, respectively, compared with those of PACS and PAD, (7.04, 8.15) and (6.68, 7.90) μg/g, respectively. The same trend was observed in males (Table 6) (P > 0.001).

Discussion

This study provides data on the essential trace element (Cu, Cr Fe, Mn and Zn) and TEs (Cd, Ni and Pb) in scalp hair samples obtained from smoker and alcohol user diabetics and referents subjects of both genders of age group (35–55 years).

Trace elements are uniquely required for growth and maintenance of life and health. Lack or an inadequate supply of such nutrients produces a functional impairment or can result in disease [31]. There is accumulating evidence that the metabolism of several trace elements is altered in insulin-dependent DM and that these nutrients might have specific roles in the pathogenesis and progress of this disease [32].

Tobacco use has long been known to be a major risk factor for cardiovascular disease, and recent study has identified a positive association between smoking and incidence of diabetes [33]. The evidence that smoking is an independent risk factor for the development of diabetes is still considered preliminary [34]. Drinking too much alcohol can raise the levels of some fats in the blood (triglycerides). It can also lead to high blood pressure, heart failure, diabetes and an increased calorie intake. Some studies have shown a dose–response association between smoking and incidence of diabetes [33, 34]. Also, some earlier prospective research failed to find an increased risk of diabetes among tobacco users [34].

Several hypotheses have been proposed to link tobacco use and incidence of diabetes. Smoking has been linked to impaired response to glucose tolerance tests and insulin resistance [35]. Although smoking cessation can result in modest weight gain, smoking is related to a more unhealthy distribution of upper body weight and greater waist:hip ratio [36]. Smoking and dinking alcohol have also been associated with risk of chronic pancreatitis and pancreatic cancer, suggesting that tobacco smoke may be directly toxic to the pancreas [37]. Tobacco smoke, which exists in two major phases, namely the gas phase and particulate (tar) phase, has a large number of chemical carcinogens and generates reactive oxygen species, which can lead to oxidative stress in the lung and other organs. The carcinogens, oxidants, and a number of toxic substances have direct or indirect, modulatory or damaging effects on DNA, membrane lipids, cell signaling proteins, and various macromolecules [38].

People with diabetes are more likely to have high blood pressure and high levels of fats such as triglycerides. Several factors, including genetics and obesity, increase a person's risk of insulin resistance and smoking has also been shown to increase the risk of this condition. It is believed that catecholamines, are produced in greater quantity in smokers and act as an antagonist to insulin action [39].

Tobacco leaves naturally accumulate and concentrate relatively high levels of Cd, Ni, Pb, Fe, Cu, and therefore smoking of tobacco is an important source of these metals exposure for smokers [40]. The total amount of metals carcinogens in cigarette smoke ranges from 1 to 3 μg per

cigarette [41]. The country of origin and type of the product play major roles in determining the chemical composition of cigarette tobacco [20]. It was investigated that one pack of cigarettes deposits 2–4 μg Cd, 1–2 μg Pb and 0.96–1.34 μg Ni into the lungs of a smoker, whereas some of the smoke passes into the air to be inhaled by smokers and nonsmokers alike [40]. It was also consistent with another study that smokers generally exhibit significantly higher Cd, Ni, Pb, Fe and Cu body burdens than non-smokers, while smoking with alcohol consumption enhance the Cd, Ni, Pb, Fe and Cu absorption and accumulation in all the tissues [23]. The results suggested that although these toxic elements (Cd, Ni, Pb) pose a hazard to essential trace metal homeostasis of various organs, co-exposure can pose a major threat, while consumption of ethanol may absorb much more Cd and Pb than their unexposed counterparts [42].

This study revealed that the level of Zn was low in scalp hair samples of diabetic smokers and alcohol consumers. Alteration of Zn homeostasis in diabetics is supported by a large body of experimental and clinical evidence. The low levels of Zn in diabetic patients may be due to excessive urinary output especially in patients with diabetic nephropathy or signs of infection during which Zn will act as a defense mechanism [43]. In diabetic individuals, enteric neuropathy and microvascular disease can alter intestinal absorption of carbohydrates, amino acids, and minerals [44]. Zinc deficiencies in diabetics are associated with excess free radical activity, and the increased oxidation of fats (lipids). When fats become oxidized, they are believed to become more reactive and damaging to the heart, arteries, and other integral parts of the vascular system [7].

The concentration of copper and iron were higher in the scalp hair samples of smokers and alcohol drinker diabetic patients as compared to referents of both genders (Table 6). Excess iron has been implicated in the pathogenesis of diabetes and its complications [45]. Free iron serves as a catalyst for lipid and protein oxidation and the formation of reactive oxygen species. In addition, iron indices are correlated with obesity and insulin sensitivity [46]. In the presence of hyperglycemia and inflammation, iron may contribute towards the development and progression of oxidative injury. Iron may also negatively impact on glycemic control [16]. However, iron indices are strongly correlated with Hb, which represents an important risk factor for morbidity and mortality in patients with diabetes, particularly in patients with established cardiovascular disease [47]. Increase in Cu concentration has been linked with disorders in the structure of the arterial walls, stress, and infection and also in diabetes mellitus [48]. The relationship between an increase in Cu concentration and the oxidation of low-density lipoproteins has been verified [49].

Concentration of Cr in the scalp hair samples of diabetic subjects were found to be significantly lower than referents ($p > 0.001$). Our results are consistent with other investigators, Anderson; 2002, 1998 [50, 51] who elucidated the action of Cr in diabetes and showed that the administration of Cr may have beneficial effects on the disease. Cr is an essential element required for normal carbohydrate and lipid metabolism. Many scientists have demonstrated that a sever Cr deficiency led to fasting - hyperglycemia, glucosuria and impaired growth [52]. Our older patients had lower values of Cr in scalp hair samples which is consistent with other studies [53], where age-related decreases of Cr in hair as compared to matched normal subjects were described. In general, based on observations from different groups of studies, in addition to impaired Cr utilization, age plays a major role in the status of Cr. Results from some trials [54] have indicated that Cr supplementation increases muscle gain and fat loss associated with exercise and improves glucose metabolism and the serum lipid profile in patients with or without diabetes. Most of the patients under study have cardiac problems, which is consistent with other studies, who reported that low Cr concentrations and the associated impairments in insulin, glucose and lipid metabolism resulting in increased cardiovascular risk [55, 56]. Insulin resistant diabetic patients responded well to oral doses of Mn [57]. Appropriate Mn levels are required for development of the normal insulin synthesis and secretion [58]. In our study, the diabetic patients of both genders had lower level of Mn in scalp hair samples than normal healthy groups of both genders. Recent studies with rats and humans indicate that nickel deprivation depresses growth, reproductive performance, and plasma glucose and alters the distribution of other elements in the body, including calcium, iron and zinc [59, 60].

Bonnefont-Rousselot [61] has investigated the use of minerals (vanadium, chromium, magnesium, zinc, selenium) and vitamins (tocopherol, ascorbic acid, nicotinamide, riboflavin) in diabetes, with a particular focus on the prevention of diabetic complications. It was also reported that dietary supplementation with micronutrients may be complementary to classical therapies for preventing and treating diabetic complications and the supplementation is expected to be more effective when a deficiency in these micronutrients exists [62].

The findings of the present study clearly demonstrate that the concentration of toxic metals (Cd, Pb) varied in the scalp hair samples of smoker and alcohol drinker diabetic patients as compared to smoker and referents (Table 6). Metallic carcinogenicity is generally thought to generate free radicals, and thus some metals were reported to play a role in lung tumorigenesis. The potential health impact from smoking cigarettes that delivers high levels of toxic metal is not limited to active smokers. In indoor environments, Cd, lead, arsenic and organic carcinogens from side stream smoke are readily available for passive exposure [63]. Cd

exposure from smoking cigarettes may be a more serious health concern than Cd in food. Smokers may double their daily intake of Cd compared with non-smokers. Each cigarette may contain from 1 to 2 mg of Cd, and 40–60 % of the Cd in the inhaled smoke can pass through the lungs into the body. This means that smokers may take in an additional 1–3 μg of Cd into their body per day from each pack of cigarettes smoked. Smoke from other people's cigarettes probably does not cause non-smokers to take in much Cd. Aside from tobacco smokers, people who live near hazardous waste sites or factories that release Cd into the air have the potential for exposure to Cd in air. It was reported in our previous study that the steel mill workers who smoked had significantly high level of Cd in scalp hair and blood samples as compared to the unexposed and non-smoker workers [64].

There is scarce information on Cd effects on insulin receptors and insulin action in adipose tissue. Addition of Cd (1 mM) to intact rat adipocytes did not affect the insulin receptor kinase activity, but stimulated glucose transport without changing the amount of glucose transporter in crude plasma membranes [65]. The stimulatory effect of Cd on glucose transport was also confirmed in cell culture model and again, no effects on GLUT 4 protein were observed [66]. It seems that aforementioned findings on Cd-induced glucose transport could explain previously described in vitro insulin-mimetic effect of Cd on glucose lipogenesis and glucose oxidation [66] in rat adipocytes. In pancreatic islets of obese hyperglycemic mice low Cd concentration evoked basal and glucose stimulated insulin response [67]. In contrast, high Cd concentration significantly inhibited the secretory response to glucose [67]. In vivo rat intake of Cd resulted in lower glycemia accompanied with higher serum insulin value [67]. Further discrepancies in Cd effects on glucose homeostasis and insulin levels are results of hyperglycemia and inhibition of insulin release from rat pancreas in rats exposed to Cd [67]. Incompatibility of literary data on Cd effect is based on both, experimental approach (in vivo vs. in vitro studies) and the various metal concentrations used. Low doses of Cd used in experiments mimic low or moderate levels of environmental contamination.

Lead in blood is present almost entirely in the cells. Bone lead, which comprises >95 % of adult body lead burden and has a biologic half-life ranging from years to decades, is a better biologic marker for studying chronic toxicity of accumulated exposure and lead burden [68]. In addition, bone lead also serves as an endogenous source of lead exposure for individuals with increased bone turnover [69]. Therefore, bone lead may be a risk factor for impaired renal function either by serving as a dosimeter of cumulative exposure of the kidney to lead or a measure of the major endogenous source of blood lead that, in turn, may affect the kidney. An increase in bone resorption is a characteristic of aging in both men and women, aging-associated release of bone lead into the circulation is a potentially important source of soft-tissue lead exposure and toxicity. Another factor associated with aging that may increase the nephrotoxicity of lead is diabetes. The more prevalent form, type-2 diabetes, affects approximately 10 % or more of the general population (with substantially higher rates at $\geq$ years of age) [70] and is well known as an independent predictor of accelerated decline in kidney function.

Conclusion

The results of this study revealed that diabetics have a different pattern of essential trace and toxic elements in their scalp hair samples than controls/referents, with the prevalence being more in smokers and alcohol users. However, higher levels of Cd, Cu, Fe, Pb and Ni, as well as a lower level of Cr, Mn, Zn, correlated well with the consequences of DM.

The impaired trace element metabolism of the present work may have a role in the pathogenesis and progression of DM where the increase of Fe, Cd, Cu, Ni and Pb and decrease of Zn, Cr and Mn concentration in scalp hair samples of diabetics may disturb the secretion and action of insulin, the high level of Cu, Cd, Fe and Pb may disturb the antioxidants, and enhance the lipid peroxidation. Smoking and alcohol consumption further aggravates the problem by increasing the level of toxic elements (Cd, Pb, Ni).

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References

1. Groop LC. Insulin resistance: the fundamental trigger of type 2 diabetes. Diabetes Obes Metab. 1999;1:1–7.
2. Zargar AH, Shah NA, Masoodi SR, Laway BA, Dar FA, Khan AR, Sofi FA, Wani AI. Copper, zinc and magnesium levels in type-1 diabetes mellitus. Saudi Med J. 2002;23:539–42.
3. Bhanot S, Thompson KH, Mcneill JH. Essential trace elements of potential importance in nutritional management of diabetes mellitus. Nutr Res. 1994;14:593–604.
4. Zargar AH, Shah NA, Massodi SR. Copper, zinc and magnesium levels in non- insulin-dependent diabetes mellitus. Postgard Med J. 1998;74:665–8.
5. Chen MD, Lin PY, Tsou CT, Wang JJ, Lin WH. Selected metals status in patients with noninsulin-dependent diabetes mellitus. Biol Trace Elem Res. 1995;50:119–24.
6. Chausmer AB. Zinc, insulin and diabetes. J Am Coll Nutr. 1998;17:109–15.
7. DiSilvestro RA. Zinc in relation to diabetes and oxidative disease. J Nutr. 2000;130:1509–11.

8. Singh RB, Niaz MA, Rastogi SS, Bajaj S, Gaoli Z, Shoumin Z. Current zinc intake and risk of diabetes and coronary artery disease and factors associated with insulin resistance in rural and urban populations of North India. J Am Coll Nutr. 1998;17:564–70.
9. Anderson R, Roussel AM, Zouari N. Potential antioxidant effects of zinc and chromium supplementation in people with type 2 diabetes mellitus. J Am Coll Nutr. 2001;20:212–8.
10. Al-Maroof RA, Al-Sharbatti SS. Serum zinc levels in diabetic patients and effect of zinc supplementation on glycemic control of type 2 diabetics. Saudi Med J. 2006;27:344–50.
11. Stupar J, Vrtovec M, Dolinsek F. Longitudinal hair chromium profiles of elderly subjects with normal glucose tolerance and type 2 diabetes mellitus. Metab Clin Exp. 2007;56:94–104.
12. Althuis MD, Jordan NE, Ludington EA, Wittes JT. Glucose and insulin responses to dietary chromium supplements: a meta- analysis. Am J Clin Nutr. 2002;76:148–55.
13. Bond JS, Failla ML, Unger DF. Elevated manganese concentration and arginase activity in livers of streptozotocin-induced diabetic rats. J Biol Chem. 1983;258:8004–9.
14. Korc M. Manganese action on pancreatic protein synthesis in normal and diabetic rats. Am J Physiol. 1983;245:628–34.
15. Fatima N, Maqsood ZT, Khan B. Study of some micronutrients in selected medicinal plants. Sci Iran. 2005;12:269–73.
16. Fernandez-Real JM, Penarroja G, Castro A, Garcia-Bragado F, Hernandez-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes. Effects on insulin sensitivity and β-cell function. Diabetes. 2002;51:1000–4.
17. Jiang R, Manson JE, Meigs JB. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA. 2004;291:711–7.
18. Klaus KA, Witte MB, Andrew L, Clark MA, John GF. Chronic heart failure and micronutrients. J Am Coll Cardiol. 2001;37:1765–74.
19. Tso TC. Seed to smoke. In: Davis DL, Nielsen MT, editors. Tobacco: production, chemistry and technology. Oxford: Black- well Science; 1999.
20. Tsadilas CD. Soil pH influence on cadmium uptake by tobacco in high cadmium exposure. J Plant Nutr. 2000;23:1167–78.
21. Chiba M, Masironi R. Toxic and trace-elements in tobacco and tobacco-smoke. Bull WHO. 1992;70:269–75.
22. Gairola CG, Wagner GJ. Cadmium accumulation in the lung, liver and kidney of mice and rats chronically exposed to cigarette smoke. J Appl Toxicol. 1991;11:355–8.
23. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco- induced cancer. Nat Rev Cancer. 2003;3:733–44.
24. Lin JL, Lin-Tan DT, Hsu KH, Yu CC. Environmental lead exposure and progression of chronic renal diseases in patients without diabetes. N Engl J Med. 2003;348:277–86.
25. Buchet JP, Lauwerys R, Roels H, Bernard A, Bruaux P, Claeys F. Renal effects of cadmium body burden of the general population. Lancet. 1990;336:699–702.
26. Akesson A, Lundh T, Vahter M, Bjellerup P, Lidfeldt J, Nerbrand C. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. Environ Health Perspect. 2005;113:1627–31.
27. Jin T, Nordberg G, Sehlin J, Wallin H, Sandberg S. The susceptibility to nephrotoxicity of streptozotocin-induced diabetic rats subchronically exposed to cadmium chloride in drinking water. Toxicology. 1999;142:69–75.
28. Kazi TG, Afridi HI, Kazi N, Jamali MK, Arain MB, Jalbani N, Baig JA. Distribution of zinc, copper and iron in biological samples of Pakistani myocardial infarction (1st, 2nd and 3rd heart attack) patients and controls. Clin Chim Acta. 2008;389:114–9.
29. Afridi HI, Kazi TG, Kazi N, Jamali MK, Arain MB, Jalbani N, Shah AQ. Evaluation of status of toxic metals in biological samples of diabetes mellitus patients. Diabetes Res Clin Pract. 2008;80:280–8.
30. Afridi HI, Kazi TG, Kazi GH. Analysis of heavy metals in scalp hair samples of hypertensive patients by conventional and microwave digestion methods. Spectrosc Lett. 2006;39:203–14.
31. Webb P, Nishida C, Darnton-Hill I. Age and gender as factors in the distribution of global micronutrient deficiencies. Nutr Rev. 2007;65:233–45.
32. Abou-Seif MA, Youssef AA. Evaluation of some biochemical changes in diabetic patients. Clin Chim Acta. 2004;346:161–70.
33. Will JC, Galuska DA, Ford ES, Mokdad A, Calle EE. Cigarette smoking and diabetes mellitus: evidence of a positive association from a large prospective cohort study. Int J Epidemiol. 2001;30:540–6.
34. Haire-Joshu D, Glasgow RE, Tibbs TL. Smoking and diabetes. Diabetes Care. 1999;22:1887–98.
35. Attvall S, Fowelin J, Lager I, Von Schenck H, Smith U. Smoking induces insulin resistance—a potential link with the insulin resistance syndrome. J Intern Med. 1993;233:327–32.
36. Shimokata H, Muller DC, Andres R. Studies in the distribution of body fat. III. Effects of cigarette smoking. JAMA. 1989;261:1169–73.
37. Talamini G, Bassi C, Falconi M, Sartori N, Salvia R, Rigo L. Alcohol and smoking as risk factors in chronic pancreatitis and pancreatic cancer. Dig Dis Sci. 1999;44:1303–11.
38. Pryor WA. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. Environ Health Perspect. 1997;4:875–82.
39. Cigarettes: what the warning label doesn't tell you? The American Council on Science and Health, 1996.
40. Kazi TG, Jalbani N, Arain MB. Toxic metals distribution in different components of Pakistani and imported cigarettes by electrothermal atomic absorption spectrometer. J Hazard Mater. 2009;163:302–7.
41. Csalari J, Szantai K. Transfer rate of cadmium, lead, zinc and iron from the tobacco-cut of the most popular Hungarian cigarette brands to the combustion products. Acta Aliment. 2002;31:279–88.
42. Sharma G, Sandhir R, Nath R, Gill K. Effect of ethanol on cadmium uptake and metabolism of zinc and copper in rats ex- posed to cadmium. J Nutr. 1991;121:87–91.
43. Chung JS, Franco RJS, Curi PR. Renal excretion of zinc in normal individuals during zinc tolerance test and glucose tolerance test. Trace Elem Electrol. 1995;12:62–7.
44. Eliasson B, Bjornsson E, Urbanavicius V, Andersson H, Fowelin J, Attvall S, Abrahamsson H, Smith U. Hyperinsulinaemia impairs gastrointestinal motility and slows carbohydrate absorption. Diabetologia. 1995;38:79–85.
45. Thomas MC, MacIsaac R, Tsalamandris C, Power D, Jerums G. Unrecognised anaemia and diabetes; a cross sectional survey. Diabetes Care. 2003;26:1164–9.
46. Thomas MC, MacIsaac RJ, Tsalamandris C, Jerums G. Elevated iron indices in patients with diabetes. Diabet Med. 2004;21:798–802.
47. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA. 2002;287:2570–81.
48. Beshgetoor D, Hambidge M. Clinical conditions altering copper metabolism in humans. Am J Clin Nutr. 1998;67:1017S–21.
49. Tan KCB, Aiv VGH, Chow WS, Chau MT, Leong L, Lam KSL. Influence of Low Density Lipoprotein (LDL) subfraction profile and LDL oxidation on endothelium-dependent and independent vasodilation in patients with type 2 diabetes. J Clin Endocrinol Metab. 1999;84:3212–6.
50. Anderson RA. Chromium in the prevention and control of diabetes. Diabetes Metab. 2002;26:22–7.
51. Anderson RA. Chromium, glucose intolerance and diabetes. J Am Coll Nutr. 1998;17:548–55.

52. Cefalu WT, Wang ZQ, Zhang XH, Baldor LC, Russell JC. Oral chromium picolinate improves carbohydrate and lipid metabolism and enhances skeletal muscle Glut-4 translocation in obese, hyper- insulinemic (JCR-LA corpulent) rats. J Nutr. 2002;132:1107–14.
53. Davies S, McLaren Howard J, Hunnisett A, Howard M. Age- related decreases in chromium levels in 51,665 hair, sweat, and serum samples from 40,872 patients: implications for the preven- tion of cardiovascular disease and type II diabetes mellitus. Me- tabolism. 1997;46:469–73.
54. Bahijri S, Mufti M. Beneficial effects of chromium in people with
type 2 diabetes, and urinary chromium response to glucose load as a possible indicator of status. Biol Trace Elem Res. 2002;85:97–109.
55. Khamaisi M, Wexler ID, Skrha J, Strojek K, Raz I, Milicevic Z.
Cardiovascular disease in type 2 diabetics: epidemiology, risk factors and therapeutic modalities. Isr Med Assoc J. 2003;5:801–6.
56. Rajpathak S, Rimm EB, Li T, Morris JS, Stampfer MJ, Willett WC, Hu FB. Lower toenail chromium in men with diabetes and cardio- vascular disease compared with healthy men. Diabetes Care.
2004;27:2211
–6.
57. Ekmekcioglu C, Prohaska C, Pomazal K, Steffan I, Schernthaner G, Marktl W. Concentrations of seven trace elements in different hematological matrices in patients with type 2 diabetes as com- pared to healthy controls. Biol Trace Elem Res. 2001;79:205–19.
58. Naga Raju GJ, Sarita P, Ramana Murty GAV, Ravi Kumar M, Reddy BS, Charles MJ, Lakshminarayana S, Vijayan V. Estimation of trace elements in some anti-diabetic medicinal plants using PIXE technique. Appl Radiat Isot. 2006;64:893–900.
59. Nielson FH. Individual functional roles of metal ions in vivo:
beneficial metal ions. Nickel. In: Berthon G, editor. Handbook of metal-ligand interactions in biological fluids. Bioinorganic medi- cine, vol. 1. New York: Marcel Dekker; 1995. p. 257–60.
60. Yarat A, Nokay S, Ipbuker A, Emekli N. Serum nickel levels of diabetic patients and healthy controls by AAS with a graphite furnace. Biol Trace Elem Res. 1992;35:273–80.
61. Bonnefont-Rousselot D. The role of antioxidant micronutrients in the prevention of diabetic complications. J Treat Endocrinol.
2004;3:41–52.
62. O'Dell BL, Sunde RA. Handbook of Nutritionally Essential Min- eral Elements. Marcel Dekkar; 1997.
63. Ezaki O. IIb group metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat
adipocytes. J Biol Chem. 1989;264:16118–22.
64. Harrison SA, Buxton JM, Clancy BM, Czech MP. Evidence that erythroid-type glucose transporter intrinsic activity is modulated by cadmium treatment of mouse 3T3-L1 cells. J Biol Chem.
1991;266:19438–49.
65. Yamamoto A, Wada O, Ono T, Ono H. Cadmium stimulates glucose metabolism in rat adipocytes. J Inorg Biochem.
1986;27:221–6.
66. Nilsson T, Rorsman F, Berggren PO, Hellman B. Accumulation of cadmium in pancreatic beta cells is similar to that of calcium in being stimulated by both glucose and high potassium. Biochim Biophys Acta. 1986;888:270–7.
67. Merali Z, Singhal RL. Diabetogenic effects of chronic oral cadmium administration to neonatal rats. Br J Pharmacol. 1980;69:151–7.
68. Gonzalez-Cossio T, Peterson KE, Sanin LH, Fishbein E, Palazue-
los E, Aro A. Decrease in birth weight in relation to maternal bone- lead burden. Pediatrics. 1997;100:856–62.
69. Silbergeld EK. Lead in bone: implications for toxicology during
pregnancy and lactation. Environ Health Perspect. 1991;91:63–70.
70. Ford ES. Vitamin supplement use and diabetes mellitus incidence among adults in the United States. Am J Epidemiol. 2001;153:892–7.

ORIGINAL ARTICLE

Serum nitric oxide metabolites and high sensitivity C-reactive protein are important biomarkers in non obese, Indian type 2 diabetic males

Rajlaxmi Sarangi & Somanath Padhi & Srikrushna Mahapatra & Nateshan Bhumika

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Abstract Inflammation, endothelial dysfunction, and oxidative stress are postulated to be the principal events in the pathogenesis of type 2 diabetes mellitus and its vascular complications. Serum Nitric Oxide metabolites (NO_X) and high sensitivity C-reactive protein (hs-CRP) were measured in eighty non-obese, type 2 diabetic males (40 to 65 years) without (group B, N040) and with vascular disease [16 retinopathy (group C), 24 hypertension (group D)]; and compared with forty healthy, age and sex matched control subjects (group A). The mean age of diabetic patients and healthy controls was 49.5±5.80 vs. 51.0±7.15 years, respectively (P00.212). Diabetic group had higher Fasting Plasma Glucose (FPG), hs-CRP, and NO_X levels than control group (135.3±28.69 vs. 96.7±10.46; 5.1±2.59 vs. 1.7±0.54; 61.1±15.67 vs. 37.1±3.69, respectively, P00.001). When compared with diabetics without complication (53.7±10.37), levels of NO_X were significantly higher (P0 0.001) both in retinopathy (74.5±19.39) and hypertension (64.4±13.60) group, whereas that of hs-CRP (4.4±2.85 vs.6.5±2.93, P00.015) differed only in retinopathy group. There was no difference between retinopathy and hypertension group. There was significant ($P<0.05$) positive correlation between age, duration of diabetes, hs-CRP, and NO_X; hs-CRP and NO_X among group B and C. A negative correlation was noted between hs-CRP and NO_X (r0−0.068, P0 0.752) among hypertensives. By multivariate regression analysis, FPG, hs-CRP and NO_X were found to be independent indicators of complications after adjustment for age and duration of diabetes. This study reveals that NO_X and hs-CRP are important biomarkers in type 2 diabetes mellitus and its associated vascular complications.

Keywords Nitric oxide metabolites · hs-CRP · Retinopathy · Hypertension · Type 2 diabetes mellitus

S. Padhi (✉)
somanath.padhi@gmail.com

Introduction

Hyperglycemia is considered as a primary cause of diabetic vascular complications and is associated with oxidative stress, and vascular inflammation [1–3]. High sensitivity C- reactive protein (hs-CRP), a marker of systemic inflammation, has been shown to be an important biomarker in predicting the major adverse cardiovascular events (MACE) in patients with type 2 diabetes [4–8]. Diabetic Retinopathy (DR), a leading cause of blindness in the Western world, has been shown to be a vascular-neuroinflammatory disease resulting due to oxidative stress, vascular inflammation, and cytokine mediated microglial activation [9]. Inactivation of endothelial Nitric Oxide Synthase (eNOS) in type 2 diabetes is responsible for decreased bioavailability of Nitric Oxide (NO), increased reactive oxygen species (ROS), lipid peroxidation, and consequent neointimal proliferation;

leading to vascular complications [10, 11]. In addition to metabolic stress of hyperglycemia, hypertension and dyslipidemia are known to influence the NO production [12].

The objective of the present study is to compare the serum levels of NO derived metabolites (Nitrate + Nitrite, NO_X) and hs-CRP in patients with type 2 diabetes mellitus; and test the hypothesis that 'inflammation and inflammatory markers possibly contribute to diabetogenesis and its vascular complications.'

Materials and methods

Following the approval of Institutional Ethics Committee, a prospective case control study was conducted in the Department of Biochemistry of our Institute over a period ranging from December 2008 to June 2010. Informed consent was obtained in all subjects and the outcome of the analyses was kept confidential.

This study included one hundred and twenty non obese (body mass index < 25 kg/m^2) males between 40 and 65 year of age comprising of 40 healthy, age and sex matched control subjects from general population (group A), 40 well controlled (with diet, exercise, and drugs) type 2 diabetic patients without any known complication (group B), and 40 type 2 diabetics with complication (group C and D). Group C comprised of 16 patients with retinopathy (microvascular disease), diagnosed on the basis of fundoscopic examination and fluorescein angiography; whereas 24 patients with systemic hypertension (macrovascular disease), with or without medication, constituted group D. As per World Health Organization (WHO) criteria, normal blood pressure was defined as Systolic Blood Pressure (SBP) <140 mmHg and Diastolic Blood Pressure (DBP) <90 mmHg. Hypertension was defined as either SBP ≥160 mmHg or DBP of ≥95 mmHg, or both, with a well-documented long term history [12]. Subjects (both control and patients) with present or past history of smoking, alcohol consumption, MACE, acute or chronic inflammatory disorders (upper or lower respiratory infection, bronchial asthma), urinary tract infection, inflammatory bowel disease, osteoarthritis, rheumatoid arthritis, gout, hepatitis), malignancy were excluded from the present study [4].

Following an overnight fast (12 h), 5 ml of blood sample was collected from anterior cubital vein under aseptic condition in all subjects. One ml was kept in fluoride for estimation of glucose. The remainder of the blood sample was kept in a plain dry sterile vial without any anticoagulant and allowed to clot. After retraction of the clot, serum was separated by centrifugation at 3,000 rpm for 10 min. Fasting Plasma Glucose (FPG) was estimated by commercially available kits (Glucose Oxidase-Peroxidase method). Diabetes mellitus was defined as FPG of ≥ 126 mg/dL or as receiving anti-hyperglycemic drug treatment [13]. Levels of serum NO_X were measured using Griess chemical method in a spectrophotometer at 540 nm [14]. Levels of hs-CRP (microgram per millilitre) were measured using enzyme linked immunosorbent assay on micro titre well (ELISCAN Catalogue No. CRO16CM).

Quantitative data were presented as mean ± Standard Deviation (SD). Student T test was performed to determine the difference between variables between groups. Pearson correlation coefficient (r) and multivariate regression analyses were used to assess the association between variables. P value less than 0.05 was considered statistically significant. SPSS 16 was used for analyses.

Table 1 Comparison of hs-CRP and NO_x between control group (A) and all patients of type 2 diabetes mellitus (B+C+D)

Parameters	Group A (N040)	Group B+C+D (N080)	T value	P* value
Age (years)	51.0±7.15	49.5±5.80	1.254	0.212
FPG (mg/dl)	96.7±10.46	135.3±28.69	−8.232	0.001
hs-CRP (mcg/ml)	1.7±0.54	5.1±2.59	−8.252	0.001
NO_X (micromol/L)	37.1±3.69	61 .1±15.67	−9.517	0.001

A control group, B Diabetes without complications (N0 40), C Diabetic retinopathy (N0 16), D Diabetes with hypertension (N0 24), FPG fasting plasma glucose in milligram per decilitre, hs-CRP high sensitivity C-reactive protein in microgram per millilitre, NO_X nitric oxide metab- olite in micromole per litre, * P value less than 0.05 is considered significant

Results

The mean age of diabetic patients and healthy controls, in the present study, was 49.5±5.80 vs. 51.0±7.15 years, respectively (P00.212). Diabetic patients had significantly

Table 2 Comparison of hs-CRP and NO_x between the patients of type 2 Diabetes mellitus without (Group B) and with complications (Group C+D)

Parameters	Group B (N040)	Group C+D (N040)	T value	P* value
Age (years)	48.8±5.48	50. 1±6.10	1.041	0.301
FPG (mg/dL)	134.3±27.80	136.4±29.88	0.322	0.749
Duration of diabetes (years)	5.2±2.53	6.4±2.45	2.061	0.043
hs-CRP (mcg/mL)	4.4±2.85	5.8±2.13	2.394	0.019
NO_X (micromol/L)	53.7±10.37	68.5±16.69	4.750	0.001

B Diabetes without complications (N0 40), C Diabetic retinopathy (N0 16), D Diabetes with hypertension (N0 24), FPG fasting plasma glucose in milligram per decilitre, hs-CRP high sensitivity C - reactive protein in microgram per millilitre, NO_X nitric oxide metabolite in micromole per litre, * P value less than 0.05 is considered significant

Table 3 Comparison of hs-CRP and NO_x between patients of type 2 diabetes mellitus without complication (Group B), with retinopathy (Group C), and with hypertension (Group D)

Parameters	Group B N0 40	Group C N0 16	T value	P* value	Group D N0 24	T value	P* value
Age (years)	48.8±5.48	51.5±7.13	−1.510	0.137	49.2±5.28	−0.334	0.739
FPG	134.3±27.80	128.8±21.31	0.704	0.484	141.4±33.94	−0.909	0.367
Duration of diabetes (years)	5.2±2.53	6.8±2.75	−2.084	0.042	6.1±2.25	−1.354	0.181
hs-CRP	4.4±2.85	6.5±2.93	−2.507	0.015	5.2±1.16	−1.346	0.183
NO_X	53.7±10.37	74.5±19.39	−5.206	0.001	64.4±13.60	−3.564	0.001

N number of subjects, FPG fasting plasma glucose in milligram per decilitre, hs-CRP high sensitivity C-reactive protein in microgram per millilitre, NO_X nitric oxide metabolite in micromole per litre, * P value less than 0.05 is considered significant

higher (P00.001) levels of FPG (135.3±28.69 vs. 96.7± 10.46), hs-CRP (5.1±2.59 vs. 1.7±0.54), and NO_X (61 .1± 15.67 vs. 37.1±3.69) (Table 1).

Compared to diabetics without complication (group B), those with vascular disease (group C and D) had longer duration of diabetes (5.2±2.53 vs. 6.4±2.45 years, P00.043), higher level of hs-CRP (4.4±2.85 vs. 5.8±2.13, P00.019), and NO_X (53.7±10.37 vs. 68.5±16.69, P00.001) (Table 2).

Compared to group B (Table 3), retinopathy group (C) had longer duration of diabetes (5.2±2.53 vs. 6.8±2.75, P0 0.042, r0−2.084), higher hs-CRP (4.4±2.85 vs. 6.5±2.93, P00.015), and NO_X (74.5±19.39 vs. 53.7±10.37, P 0
0.001). However, there was no difference between these two groups in relation to age (P00.137) and FPG (P0 0.484). However, none of the parameters except NO_X (53.7±10.37 vs.64.4±13.60, P00.001) was found to be significant among diabetic hypertensives (group D). There was no difference between diabetics, either with retinopathy or with hypertension in relation to any of the studied parameters (Table 4).

Among diabetics without any vascular complications (group B), significant (P00.001) positive correlation was noted between age with duration of diabetes (r00.517), hs-CRP (r00.563), and NO_X (r00.645); duration of diabetes with hs-CRP (r00.401, P00.010) and NO_X (r00.427, P0 0.006); hs-CRP and NO_X (r00.879, P00.001). Similar were the observations among retinopathy group (group C) [age and duration of diabetes (r00.750, P00.001), hs-CRP (r00.685, P00.003), and NO_X (r00.713, P00.002); hs-CRP and NO_X (r00.935, P00.001)]. Hypertensive diabetics (group D) showed significant positive correlation of age with duration of diabetes (r00.461, P00.024) and hs-CRP (r00.668, P0 0.001), but insignificant correlation with NO_X (r00.071, P0 0.740), hs-CRP with NO_X (r0−0.068, P00.752). Duration of diabetes had a positive correlation with both hs-CRP (r0 0.401, P00.001) and NO_X in group B; with hs-CRP in group D (r00.466, P00.022); but insignificant correlation with both in group C. Fasting plasma glucose had no significant correlation with any of the other three variable studied in any of the diabetic group (P>0.05) (Table 5).

Multivariate regression analysis was employed to study the relation between FPG, hs-CRP and NO_X and vascular compli- cations of diabetes. The independent variables were FPG, hs- CRP and NO_X, and the dependent variable was the presence of diabetic vascular complications (diabetic retinopathy and hy- pertension). Age of subjects and duration of diabetes were the control variables. Employing a 0.05 criterion of statistical significance, all three independent variables (FPG, hs-CRP and NO_X) emerged as significant markers of vascular compli- cations associated with type 2 diabetes mellitus (Table 6).

Table 4 Comparison of hs-CRP and NO_X between patients of type 2 diabetes mellitus with retinopathy (Group C) and those with hyperten- sion (Group D)

Parameters	Group C (N0 16)	Group D (N0 24)	T test	P* value
Age (years)	51.5±7.13	49.2±5.28	1.124	0.268
FPG	128.8±21.31	141.4±33.94	−1.312	0.197
Duration of Diabetes (years)	6.8±2.75	6.1±2.25	0.944	0.351
hs-CRP	6.5±2.93	5.2±1.16	1.974	0.056
NO_X	74.5±19.39	64.4±13.60	1.927	0.062

N number of subjects, FPG fasting plasma glucose in milligram per decilitre, hs-CRP high sensitivity C-reactive protein in microgram per millilitre, NO_X nitric oxide metabolite in micromole per litre, * P value less than 0.05 is considered significant

Discussion

In the present study, both hypertensive diabetics and retinopathy group had significant increase in NO_X levels than those without any complication. The level of hs-CRP was found to be high in diabetics than control, highest among retinopathy group. This was a reflection of increased inflammation and NO derived free radical mediated complications associated with increased duration of diabetes. The level of hs-CRP in diabetic hypertensive patients was not found to be significantly different as compared to those without complications (Tables 2 and 3).

Table 5 Correlation between age, fasting plasma glucose, duration of diabetes, and hs-CRP among three groups of patients with type 2 diabetes mellitus

	Variable 1	Variable 2	r$ value	P* value
Group B (Without complications, N040)	Age	FPG	0.243	0.131
		Duration of DM	0.517	0.001
		hs-CRP	0.563	0.001
		NO_X	0.645	0.001
	FPG	Duration of DM	−0.008	0.962
		hs-CRP	0.009	0.954
		NO_X	0.186	0.250
	Duration of DM	hs-CRP	0.401	0.010
		NO_X	0.427	0.006
	hs-CRP	NO_X	0.879	0.001
Group C (With retinopathy, N016)	Age	FPG	−0.188	0.486
		Duration of DM	0.750	0.001
		hs-CRP	0.685	0.003
		NO_X	0.713	0.002
	FPG	Duration of DM	−0.083	0.759
		hs-CRP	0.203	0.452
		NO_X	0.174	0.520
	Duration of DM	hs-CRP	0.459	0.074
		NO_X	0.454	0.078
	hs-CRP	NO_X	0.935	0.001
Group D (With hypertension, N024)	Age	FPG	0.246	0.247
		Duration of DM	0.461	0.024
		hs-CRP	0.668	0.001
		NO_X	0.071	0.740
	FPG	Duration of DM	0.401	0.052
		hs-CRP	0.194	0.364
		NO_X	0.315	0.134
	Duration of DM	hs-CRP	0.466	0.022
		NO_X	0.181	0.397
	hs-CRP	NO_X	−0.068	0.752

FPG fasting plasma glucose in milligram per decilitre, DM type 2 diabetes mellitus, hs-CRP high sensitivity C-reactive protein in micro- gram per millilitre, NO_X nitric oxide metabolite in micromole per litre,
$ Pearson's correlation coefficient, * P value less than 0.05 is considered significant

Table 6 Multivariate regression analysis to assess the significance of variables in patients of type 2 diabetes mellitus after adjustment for age and duration of diabetes

Independent variables	F value	P value
FPG	26.268	<0.01
hs-CRP	4.811	<0.05
NO_x	10.659	<0.01

FPG fasting plasma glucose, hs-CRP high sensitivity C-reactive protein, NO_x Nitric Oxide derived species

Nitric Oxide is a key endothelium derived molecule that plays a pivotal role in vascular homeostasis. Both metabolic (hyperglycemia, dyslipidemia) and hemodynamic stress (hypertension) are known to cause structural and functional alteration in vascular endothelium leading to impaired eNOS enzyme activity and reduced bioavailability of NO [10, 12]. Studies have shown that hypertension impairs endothelium-dependent dilation of rat coronary arteries as a result of superoxide anion mediated degradation of NO [15].

Studies relating the NO derived metabolites to hypertension have largely been equivocal in the sense that many have reported their inverse correlation [12, 15–17]; whereas others demonstrated a positive or nonsignificant correlation [18, 19]. In a recent Indian study from Jammu and Kashmir [12], the plasma concentration of NO, by using liquid chromatography, was found to be significantly lower in both essential hypertensive patients and diabetic patients without complications as compared to the healthy controls. Studies conducted on hypertensive patients have shown decreased levels of NO_X compared to normotensive controls, and antihypertensive agents such as calcium channel blockers or Angiotensin Converting Enzyme (ACE) inhibitors were effective in restoring the normal levels [16, 17]. Li et al., reported a positive association between NOx and blood pressure in normotensive African Americans who carry the "a" allele of eNOS4 polymorphism [18]. A study from Karachi also reported significant increase in NO_X in diabetics with hypertension compared to normotensive controls, but the levels were not significantly different in patients with and without hypertension. A significantly high (P00.001) serum NO_X levels among diabetic hypertensives, in our study, was in accordance with others [19]. The high levels of serum NO_X levels, in the present study, need to be interpreted with caution in the presence of hypertension and usage of antihypertensive drugs.

Ghose et al. [10] reported a low serum NO (NO_2^-) levels from a series of type 2 Indian diabetes patients by using the Griess chemical reaction, but the levels were not compared among various subgroups. Studies by various authors [3, 20–23] have shown high NO metabolites in diabetics with or without microvascular complications. Researchers in Taiwan [24] assessed the NO levels in aqueous humor and plasma using the chemiluminescence assay and observed no significant differences between any of the diabetic subgroups in the plasma NO levels. A non significant increase in level of NO derived metabolites among diabetics compared to non diabetics was also reported by Khan et al. from Karachi [19]. Our observation among diabetics, especially in retinopathy group, was in accordance with most of the above said

reports, and supports the hypothesis that NO overproduction affects insulin's metabolic action.

Results from a different study conducted by Lim et al. [25] were in discord with the trends in the literature in the sense that patients with diabetes who had higher levels of CRP and body mass index (BMI) were less likely to have retinopathy. Tsunoda et al. [26] studied the clinical significance of serum hs-CRP among 114 Japanese patients with type 2 diabetes mellitus with or without long term complications. The hs-CRP level in normotensive diabetic patients without retinopathy was not significantly different from that of normal control subjects after adjustment for age and BMI. The hs-CRP level was significantly high in the patients with hypertension, despite the presence or absence of diabetes. On the other hand, the hs-CRP level of the diabetic patients complicated with retinopathy was low; especially those with hypertension. The frequency of patients having an hs-CRP value above 1.0 milligram per litre, a value thought to be a risk factor for cardiovascular disease, was also high in the patients complicated with hypertension. But this frequency was low among diabetics with retinopathy. These results indicate that presence or absence of hypertension and retinopathy should be taken into consideration for correct interpretation of serum hs-CRP level in diabetic patients. In the present study, the level of hs-CRP was found to be highest among retinopathy group (P0 0.015) compared to those without any complication. However, no significance was found in relation to the presence of hypertension (group D, P00.183).

A very significant (P00.001) correlation was observed between hs-CRP and NO_X in diabetics without complication, and among retinopathy group (Table 5). All three parameters such as FPG, hs-CRP, and NO_X were found to be important indicators of diabetic vascular complications after adjustment for age and duration of diabetes. Our results were in accordance with existing literature that low grade systemic inflammation may predict future development of micro and macrovascular complications of type 2 diabetes [4–7, 9, 27]. Shahid et al. [15], reported a significant negative correlation between serum nitric oxide, serum glucose and HbA1c levels in diabetic hypertensive patients. Similarly, we did not observe any significant positive correlation between FPG and hs-CRP or NO_X levels in any of the diabetic subgroups. This may possibly be explained by the critical role of HbA1c in abnormal NO metabolism and vice versa. A non-significant (P0 0.752) correlation between hs-CRP and NO_X among diabetic hypertensive patients was another important finding in our study. In view of the compounding effect of hemodynamic stress (hypertension), both on the levels of hs-CRP and NO_X, our results require careful interpretation [10, 12, 25, 26]. The outcome of analysis might also vary depending upon the assay technique used and the sample selected for analysis.

To conclude, both serum NO_X and hs-CRP were found to be important biomarkers in patients with type 2 diabetes mellitus. However, the present study had few drawbacks such as relatively small number of patients with retinopathy, lack of correlation of NO_X level with usage of antihypertensive drugs, lipid parameters, and gender bias. Besides these, use of different assay techniques [spectrophotometry (in our series), chemiluminescence, or liquid chromatography] for the measurement of NO and its metabolites levels from different samples (plasma, serum, urine, aqueous humor) probably explains the interobserver variability among researchers globally. The possible role of eNOS enzyme gene polymorphism in our population needs to be studied. The outcome of the present study should be further confirmed by larger cross sectional studies involving various ethnic groups. This will help develop targeted therapies in the prevention and management of diabetic vascular complications [27].

References

1. Pitocco D, Zaccardi F, Di Stasio E, Romitelli F, Santini SA, Zuppi C, et al. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud. 2010;7:15–25.
2. Goycheva P, Gadjeva V, Popov V. Oxidative stress and its complications in diabetes mellitus. Trakia J Sci. 2006;4:1–8.
3. Abou-Seif MA, Youssef AA. Evaluation of some biochemical changes in diabetic patients. Clin Chim Acta. 2004;346:161–70.
4. Lee S, Kim IT, Park HB, Hyun YK, Kim YJ, Sung SO, Kim H. High-sensitivity C—reactive protein can predict major adverse cardiovascular events in Korean patients with type 2 diabetes. J Korean Med Sci. 2011;26:1322–7.
5. Wen J, Liang Y, Wang F, Sun L, Guo Y, Duan X, et al. C reactive protein, gamma-glutamyltransferase and type 2 diabetes in a Chinese population. Clin Chim Acta. 2010;411:198–203.
6. Bruno G, Fornengo P, Novelli G, Panero F, Perotto M, Segre O, et al. C-reactive protein and 5-year survival in type 2 diabetes: the Casale Monferrato Study. Diabetes. 2009;58:926–33.
7. Kang ES, Kim HJ, Ahn CW, Park CW, Cha BS, Lim SK, Kim KR, Lee HC. Relationship of serum high sensitivity C-reactive protein to metabolic syndrome and microvascular complications in type 2 diabetes. Diabetes Res Clin Pract. 2005;69:151–9.
8. Vikram NK, Misra A, Pandey RM, Dwivedi M, Luthra K, Dhingra V, et al. Association between subclinical inflammation & fasting insulin in urban young adult north Indian males. Indian J Med Res. 2006;124:677–82.
9. Zhang W, Liu H, Al-Shabrawey M, Caldwell RW, Caldwell RB. Inflammation and diabetic retinal microvascular complication. J Cardiovasc Dis Res. 2011;2:96–103.
10. Ghose A, Sherpa ML, Bhutia Y, Pal R, Dahal S. Serum nitric oxide status in patients with type 2 diabetes mellitus in Sikkim. Int J App Basic Med Res. 2011;1:31–5.
11. Toutouzas K, Riga M, Stefanadi E, Stefanadis C. Asymmetric dimethylarginine (ADMA) and other endogenous nitric oxide synthase (NOS) inhibitors as an important cause of vascular insulin resistance. Horm Metab Res. 2008;40:655–9.
12. Ayub SG, Ayub T, Khan SN, Dar R, Andrabi KI. Reduced nitrate level in individuals with hypertension and diabetes. J Cardiovasc Dis Res. 2011;2:172–6.
13. American Diabetes Association. Standards of medical care in diabetes—2011. Diabetes Care. 2011;34 Suppl 1:S11–61.

14. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolite in biological samples. Free Radic Biol Med.
2007;43:645–
57.
15. Shahid SM, Mahboob T. Correlation between Glycosylated He- moglobin (HbA1c) and Serum Nitric Oxide (NO). Aust J Basic Appl Sci. 2009;3:1323–7.
16. Lyamina NP, Dolotovskaya PV, Lyamina SV, Malyshev IY, Manukhina EB. Nitric oxide production and intensity of free radical processes in young men with high normal and hypertensive blood pressure. Med Sci Monit. 2003;9:CR304–10.
17. Takase H, Sugiyama M, Nakazawa A, Sato K, Ueda R, Dohi Y.
Long-term effect of antihypertensive therapy with calcium antag- onist or angiotensin converting enzyme inhibitor on serum nitrite/ nitrate levels in human essential hypertension. Arzneimittelfor- schung. 2000;50:530–4.
18. Li R, Lyn D, Lapu-Bula R, Oduwole A, Igho-Pemu P, Lankford B, et al. Relation of endothelial nitric oxide synthase gene to plasma nitric oxide level, endothelial function and blood pressure in Afri- can Americans. Am J Hypertens. 2004;17:560–7.
19. Khan MA. Nitric oxide and lipid peroxidation status in diabetes mellitus and hypertension. PhD thesis, University of Karachi, Karachi. ID Code: 1134.
20. Ozden S, Tatlipinar S, Biçer N, Yaylali V, Yildirim C, Ozbay D, et al. Basal serum nitric oxide levels in patients with type 2 diabetes
mellitus and different stages of retinopathy. Can J Ophthalmol.
2003;38:393–6.
21. Apakkan S, Ozmen B, Ozmen D, Parildar Z, Senol B, Habif S, et al. Serum and urinary nitric oxide in type 2 diabetes with or without microalbuminuria: relation to glomerular hyperfiltration. J Diabetes Complicat. 2003;17:343–8.
22. Izumi N, Nagaoka T, Mori F, Sato E, Takahashi A, Yoshida A.
Relation between plasma nitric oxide levels and diabetic retinop- athy. Jpn J Ophthalmol. 2006;50:465–8.
23. Asl SZ, Ghasemi A, Azizi F. Serum nitric oxide metabolites in
subjects with metabolic syndrome. Clin Biochem.
2008;41:1342–
97.
24. Tsai DC, Chiou SH, Lee FL, Chou CK, Chen SJ, Peng CH, et al.
Possible involvement of nitric oxide in the progression of diabetic retinopathy. Ophthalmologica. 2003;217:342–6.
25. Lim LS, Tai ES, Mitchell P, et al. C-reactive protein, body mass index, and diabetic retinopathy. Invest Ophthalmol Vis Sci.
2010;51:4458–63.
26. Tsunoda K, Arita M, Yukawa M, et al. Retinopathy and hypertension affect serum high-sensitivity C-reactive protein levels in type 2 diabetic patients. J Diabetes Complications.
2005;19:123–7.
27. Liou GI. Diabetic retinopathy: role of inflammation and potential therapies for anti-inflammation. World J Diabetes. 2010;1:12–8.

ORIGINAL ARTICLE

Investigation of Shore meter in assessing foot sole hardness in patients with diabetes mellitus - a pilot study

R. Periyasamy & Sneh Anand & A. C. Ammini

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Abstract Understanding the biomechanical properties of the diabetic foot may detect the foot at risk of ulceration. Increased foot sole hardness and the peripheral neuropathy are suggested to be important risk factors for foot ulceration, in subject with diabetes. Therefore this study was to investigate the feasibility of measuring and variation of foot sole hardness in patients with diabetes. Assessment was performed on 30 subjects aged from forty to seventy years, classified into three groups: 10 subjects with diabetic neuropathy (Group A), 10 subjects without diabetic neuropathy (Group B) and 10 non-diabetes subjects without neuropathy (Group C). Subjects underwent an assessment of loss of protective sensation (LOPS) by means of a 10 gm Semmes Weinstein monofilament and measurement of foot sole hardness by means of a durometer (ASTM-D 2240) or shore meter in eight foot sole areas. Imperceptions of the 5.07 / 10 gm Semmes-Weinstein monofilament at four or more of the eight foot sole areas was considered the threshold for neuropathy. Data was analyzed using ANOVA to detect significant difference between the groups and also Dunnett's pair wise multiple comparison t-tests was used to compare Groups A and B against the control mean (Group C). Our result shows statistical significant ($P < 0.05$) differences between Groups A and C in all foot areas, except area 6. Also significant differences were found in areas 5, 7 and 8 while comparing Groups B and C in both feet. We conclude that difference and variation in foot sole hardness were found to be significantly different in subjects with and without diabetic neuropathy using shore meter. Hence shore meter was more sensitive and provided a feasible means of measuring foot sole hardness in subjects with diabetic neuropathy. Foot sole hardness in diabetic feet can be considered as potential determinant of the foot sole ulcerations.

Keywords Diabetic neuropathy · Foot sole hardness · Loss of protective sensation · 10gm Semmes Weinstein monofilament · Shore meter

R. Periyasamy (✉)
e-mail: periyasamy25@gmail.com

Introduction

Diabetes mellitus (DM) is recognized as a major health problem in the world. Diabetic foot ulceration is one of the well-recognized long-term complications afflicting 15 % of the diabetic patients [1]. Several protocols are available for detecting the foot at risk. Loss of protective sensation (LOPS) to 10gm Semmes Weinstein monofilament (SWMF) has been recognized as a very well-known parameter to detect the foot at risk [2, 3] and recommended for regular screening of neuropathy status by the International Diabetes Federation (IDF), the World Health Organization (WHO) and National Institute for Health & Clinical Excellence (NICE) [4–7]. Another major key factor in the formation of neuropathic ulceration is believed to be hyperkeratosis (i.e. increase in foot sole hardness) arising in areas of increased foot peak pressure [8]. However, the ulcer risk threshold was undefined in these studies [9–13] as there is no absolute threshold. However ulcers develop even below the accepted high level of peak plantar pressures [10, 14].

The Durometer or shore meter is the international standard device for hardness measurement of various non-metallic materials including rubber, plastic and wood [8, 15]. Hence measurement of foot sole hardness using a Durometer or

Shore meter could prove to be a useful parameter which shows the degree of hypertrophy of the cells (callus formation) in response to abnormal plantar pressure changes resulting in formation of foot sole ulcer [16, 17]. Therefore measurement of foot pressures distribution and hardness of the foot sole may provide a threshold level to detect the foot at risk for ulcer formation.

In earlier studies, Brink [18] measured hardness using durometer only at the metatarsal heads and it is difficult to determine whether the increased hardness in diabetes was a consequence of healing following previous ulceration. However, Piaggesi et al. [8] specifically measured hardness using durometer at specific points on the foot and lower limb (heel, median of the foot, lateral foot and posterior mid-calf) and compared the feet of subjects with and without diabetic neuropathy to the control group. Recently Charanya et al. [17] and Thomas et al. [19] used durometer or shore meter to determine the foot sole hardness in patients with diabetic neuropathy. They reported that there is increase in hardness value at ulcer sites compared to controls and non-ulcerated areas. However they have focused on ulcer sites of the feet but failed to show subtle differences in hardness value between subjects with and without diabetic neuropathy as compared to control group in eight anatomical foot sole areas.

Numerous authors [15, 20–23] reported that assessment of foot sole hardness by shore meters to be accurate and reproducible in normal and pathological conditions including scleroderma, hypertrophic scars, lymphoedema and venous disease. Therefore the primary aim of this pilot study is to establish whether it is feasible to measure and quantify foot sole hardness in subjects with diabetic neuropathy, subjects without diabetic neuropathy, and subjects with healthy feet using shore meter. It is important to assess the feasibility before larger, resource intensive and time expensive studies are performed potentially using the optical pedobarograph to measure plantar pressures distribution. We hypothesize that a subject with diabetic neuropathy will have greater foot sole hardness when compared to subjects without diabetic neuropathy, compared to control group subjects in the eight anatomical foot sole areas.

Methods

In this study thirty subjects and their relatives were recruited from outpatient clinics of Biomedical and Endocrinological Lab, All India Institute of Medical Sciences, New Delhi, India. The study period was from January to March 2011. All the subjects gave written informed consent. The total 30 subjects were divided into three groups namely: (i) Group A: subjects with diabetic neuropathy, (ii) Group B: subjects without diabetic neuropathy and (iii) Group C: non-diabetic subject without neuropathy (control group). The exclusion criteria for all groups was the history of foot ulceration, presence of clinically evident peripheral vascular disease, proliferative retinopathy, scleroderma, causes of polyneuropathy other than diabetes, lymphoedema, BMI of 30 or greater. Also we have excluded the subjects who had undergone previous foot or spinal surgery or had spinal problems, because these problems may alter foot sole sensation or hardness independently of diabetes mellitus. All Subjects were weighed and their heights were measured to allow calculation of their body mass index (kg / m^2). Loss of protective sensation was assessed using 10gm Semmes-Weinstein monofilaments (SWMF). Foot sole hardness assessment was then performed using Durometer (ASTM-D 2240 standards) to calculate shore value. A questioner type datasheet regarding their demographic details, general health and diabetic management, including duration of diabetes, foot-related complications, previous history including foot-related problem was recorded. Descriptive statistics details of subjects are mentioned in Table 1. In order to simplify our analysis, we divided the foot region into different areas. In the literature, the foot is divided into ten standard significant areas as per method indicated by authors [13, 24]. For our analysis, we have divided each foot into eight areas as mentioned in Fig. 1: Medial hind foot (area 1), lateral hind foot (area 2), medial mid foot (area 3), lateral mid foot (area 4), medial fore foot (area 5), middle fore foot (area 6), lateral fore foot (area 7) and big toe (area 8).

Loss of protective sensation (LOPS) measurement using SWMF

LOPS is measured by SWMF, which exerts 10 gm force when pressed perpendicular against the skin of the sole of the foot. For testing LOPS, the subject foot was placed in a comfortable position and then 10 gm SWMF [3] is pressed perpendicular to the surface of the foot sole skin with a force just sufficient to

Table 1 Descriptive statistics details for subjects (Groups A, B and C)

	Group A DM+ N+	Group B DM+ N−	Group C DM− N−
Number of subjects (N)	10	10	10
Mean age ± SD (years)	55.8±9.6	52.2±9.1	48.5±6.5
Sex (male : female)	8 : 2	4 : 6	6 : 4
Height(m)	1.6±0.09	1.6±0.09	1.6±0.07
Weight(kg)	64.4±7.2	62.6±7.6	66.1±8.1
BMI (kg/m^2)	24.7±2.04	24.2±2.1	24.7±2.7
Type 2 diabetes(yes:no)	10 : 0	10 : 0	N/A
Duration of diabetes (years)	10.5±6.6	8.8±6.0	N/A

N/A Not applicable; DM+ Diabetes present; DM− Diabetes absent; N+ Neuropathy present; N+ Neuropathy present; N− Neuropathy absent

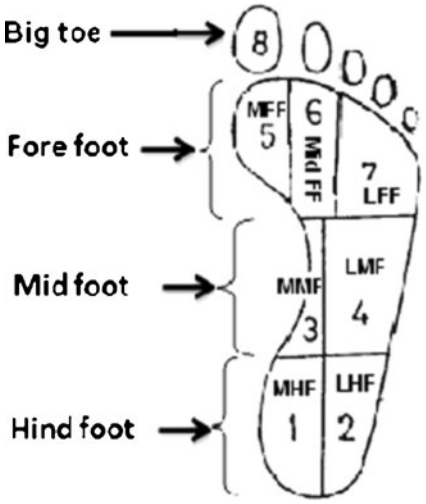

Fig. 1 Division of foot

buckle the monofilaments. Loss of sensation in more than two areas of the foot is suggestive of insensate foot.

Measurement of Hardness of foot sole using Shore meter

The instrument used for measuring the hardness of the foot sole is Shore meter or Durometer or Hardness tester (as per ASTM-D 2240 standards). This instrument is similar to the Durometer used in evaluating the hardness of foot sole soft tissue in subjects with diabetic neuropathy [8]. The Shore-meter shows the relative degree of hardness of the foot sole on a linear calibrated gauge as the result of a spring-loaded interior that senses hardness by applying an indentation load against the surface of the foot sole as mentioned in Fig. 2(a). The reading on the dial provides measure of hardness, which corresponds to the depth of indentation below the surface of the foot sole. The Shore meter reads the hardness in degree Shore (characterized as 'S'). Softer material has lower Shore value; harder material such as wood has a value of 100. Thus the Shore meter reads the Shore values from 0 to 100. The Shore meter is pre-calibrated. For measuring the hardness of the foot sole, subjects feet was held vertical and toes pointing upwards. Then Shore meter is pressed perpendicular to the surface of the foot sole areas of both the feet as shown in Fig. 2(b) and the reading indicated on the dial is noted. Three to five trials are performed at each site of foot sole as indicated in Fig. 1 and the average value noted on the datasheet. The Shore meter was never applied to ulcer sites, scar or necrotic tissue. When the Shore levels are measured on foot sole of diabetic patients, they can be any combinations of 20 to 60° shore.

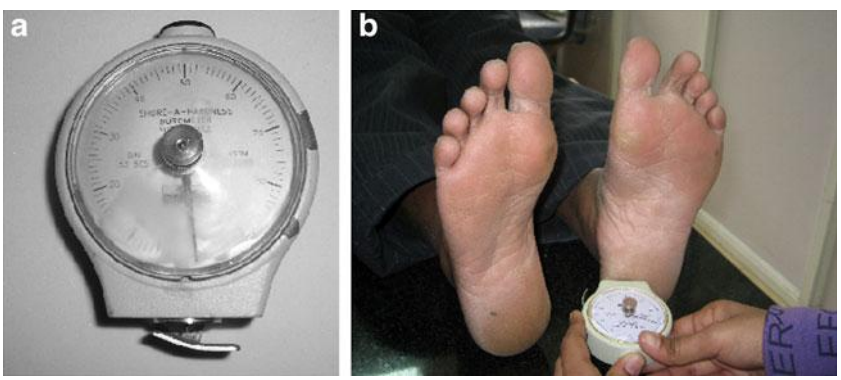

Fig. 2 a Durometer. b Hardness assessed by applying durometer on the foot

Calculation of shore indices

Foot sole hardness measured values (in degree shore) were converted to eight indices; Index 1 to Index 8. Each index was obtained by dividing measured shore value for the eight right foot areas by the corresponding areas in the left foot. As there were eight areas on the right and left foot, this yields eight indices; namely Index 1, Index 2, and Index 3, Index 4 up to Index 8.

Statistics

Data from both the right and the left foot were analyzed. The mean hardness values were compared between the groups for all foot areas as mentioned in Fig. 1. A one-way analysis of variance was then performed for each foot sole area to identify any significant difference in shore values between each group. Also Dunnett's pair wise multiple comparison t-tests were used to compare Groups A and B against the control (Group C). In addition, linear regression analysis was done to know the correlation of shore index value between the groups.

Results

In the present study, we have assessed foot sole hardness for three groups of subjects and their demographic details are summarized in Table 1. We found no statistical significant difference between the demographic details for three groups using a one-way ANOVA test.

Table 2 Shore values for the eight areas on the right foot of Group A, Group B and Group C. The ANOVA test result is recorded to assess whether there was statistically significant difference between the shore values in the three groups. Dunnett's pair wise multiple comparison t-tests are also recorded for each foot area to assess statistical significance in Groups A and B

Area	Group A (DM+ N+) Mean shore ± SD	Group B (DM+ N−) Mean shore ± SD	Group C (DM− N−) Mean shore ± SD	ANOVA
1	36.8±3.8 (P<0.05)	33.7±1.6 (ns)	29.9±4.2	P 0 0.01
2	35.6±2.7 (P<0.05)	32.9±1.9 (ns)	30.8±3.9	P 0 0.0003
3	29.2±5.0 (P<0.05)	25.5±2.1 (ns)	23.8±3.1	P 0 0.0005
4	30.9±4.6 (P<0.01)	26.9±1.5 (ns)	26.5±2.3	P 0 0.0001
5	42.8±12.0 (P<0.05)	34.4±1.5 (P<0.001)	28.2±4.1	P <0.0001
6	27.2±5.1	24.1±1.0	23.6±5.5	ns
7	34.4±5.3 (P<0.05)	30.0±2.0 (P<0.01)	27.1±2.7	P 0 0.0002
8	38.8±5.4 (P<0.05)	34.9±2.0 (P<0.005)	30.6±2.7	P <0.0001

ns not significant; DM+ Diabetes present; DM− Diabetes absent; N+ Neuropathy present; N− Neuropathy absent

Group A subjects were unable to detect the 10gm Semmes-Weinstein monofilament in two or more foot sole areas in each feet, which was considered to be due to neuropathy. However Group B subjects were able to detect the 10 gm Semmes-Weinstein monofilament in two or more foot sole areas in each foot. Similarly Group C subjects were able to detect the 10 gm Semmes-Weinstein monofilament in all foot sole areas and therefore were not considered to have plantar neuropathy.

The mean (±SD) hardness values for right and left foot estimated by the shore meter reading are presented in Tables 2 and 3. A one-way analysis of variance was then performed for each foot sole area to identify any significant difference in shore values between each group. Interestingly, all areas showed statistically significant differences in hardness values – except areas 6 in both feet. Also Dunnett's pair wise multiple compar- ison t-tests were used to compare Groups A and B against Group C. Significant differences in hardness value were found between Groups A and C for all areas in both feet, except area 6. Also significant differences in hardness value were found in areas 5, 7, 8 while comparing Groups B and C in the both feet. From Table 4 it was noted that shore index for Group A was greater in foot area 5 than Group B and C subjects but the difference was not significant (P>0.05). It appears that differences in sole tissue composition and relative thickness of foot sole soft tissues are possible factors that influence hardness values. Figure 3 show the linear regression result analysis of shore index among foot area 5 (right vs left) between Group A with Group B and C subjects

Discussion

In general, foot ulcer can develop in those patients who have had diabetes for a period of 10 to 15 years. However, occasionally, the ulcer can develop even in the earlier stages of diabetes i.e. within the first 5 years. The increase in foot

Table 3 Shore values for the eight areas on the left foot of Group A, Group B and Group C. The ANOVA test result is recorded to assess whether there was statistically significant difference between the shore values in the three groups. Dunnett's pair wise multiple comparison t-tests are also recorded for each foot area to assess statistically significant for Groups A and B

Area	Group A (DM+ N+) Mean shore ± SD	Group B (DM+ N−) Mean shore ± SD	Group C (DM− N−) Mean shore ± SD	ANOVA
1	36.8±3.9 (P<0.05)	33.7±2.3 (ns)	31.6±2.8	P <0.0001
2	33.8±3.4 (P<0.05)	30.9±2.1 (ns)	30.4±3.5	P 0 0.001
3	28.9±5.2 (P<0.01)	23.8±1.6 (ns)	23.4±2.1	P <0.0001
4	30.9±5.1 (P<0.05)	26.5±1.7 (ns)	27.9±3.3	P 0 0.006
5	37.3±6.9 (P<0.05)	32.3±1.3 (P<0.05)	29.4±4.3	P <0.0001
6	27.0±5.1	24.1±1.8	23.8±5.6	ns
7	31.2±6.6 (P<0.05)	26.7±1.3 (P<0.01)	24.2±2.3	P <0.0001
8	36.7±3.4 (P<0.05)	33.8±1.5 (p<0.05)	31.6±1.9	p<0.0001

ns not significant; DM+ Diabetes present; DM− Diabetes absent; N+ Neuropathy present; N− Neuropathy absent

Table 4 Shore indices among extremities (right foot versus left foot) in Group A, Group B and Group C subjects

Areas	Group A (DM+ N+) mean shore index ± SD	Group B (DM+ N−) mean shore index ± SD	Group C (DM− N−) mean shore index ± SD
1	1.003±0.08	1.002±0.06	0.98±0.1
2	1.07±0.1	1.06±0.09	1.04±0.14
3	1.03±0.19	1.02±0.08	1.02±0.13
4	0.99±0.16	0.96±0.08	0.96±0.08
5	1.16±0.34	1.06±0.06	0.96±0.13
6	1.02±0.21	1.01±0.1	1±0.2
7	1.13±0.20	1.12±0.1	1.12±0.09
8	1.06±0.15	1.03±0.08	0.97±0.1

DM+ Diabetes present; DM− Diabetes absent; N+ Neuropathy present; N− Neuropathy absent

sole hardness (hyperkeratosis) and loss of protective sensation is the key risk factor for neuropathic foot ulceration in patients with diabetes. In our study we used 10gm Semmes-Weinstein monofilaments as effective screening instrument [25] because of its specificity and sensitivity of 93 % and 100 % for finding neuropathy status [26] by assessing loss of protective sensation i.e. insensate two or more foot sole areas. This permits stratification of subjects into three groups.

In our study we used shore meter for the measurement of foot sole hardness in diabetes mellitus subjects and normal subjects as stated in other studies [22, 23]. Previously Brink [18] investigated ten diabetic feet with polyneuropathy and a history of recurrent plantar ulcers. They showed metatarsal heads with highest shore values recorded from Group A subjects, then Group B subjects, followed by Group C subjects with lowest shore values. But in our study we showed that shore values in Group A subjects tended to be greater than shore values in Group B subjects, which tended to be greater than values in Group C subjects in all foot sole areas. This is because foot sole hardness increases due to other factors like glycation of keratin, more cross-linked collagen chain structure independent from pressure distribution, loss of control of ankle joints and difference in footwear insole properties, foot sole tissue composition and nutrition. In agreement with our findings, Piaggesi et al. [8] found that foot sole hardness measured by a durometer was diffusely increased in Group A subjects as assessed by 10gm SWMF. In addition, they found no difference in hardness value between the Group B and Group C subjects. However, they only measured hardness value at three plantar sites (heel, medial mid-foot and lateral mid-foot) and the posterior calf as a control site. Recently Thomas et al. [19] measured skin hardness in subjects with diabetes with normal controls. They found the foot sole hardness increased at the ulcer sites compared to controls and non- ulcerated areas. But in our study we measured foot sole hard- ness for subjects with diabetes and healthy subjects in eight anatomical areas. We showed statistically significant ($P < 0.05$; $P < 0.01$) difference in the hardness values between Groups A and C in all foot sole areas except areas 6 (mid forefoot)

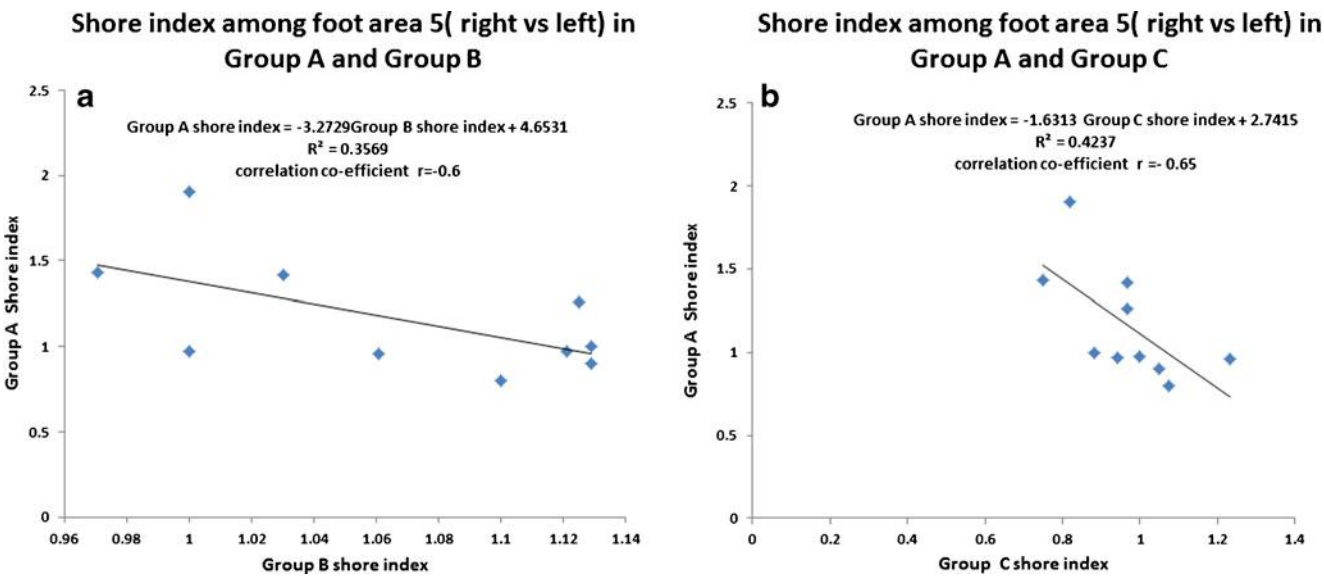

Fig. 3 Correlation of shore index among foot area 5 between Group A with group B and group C

because this area was usually not exposed to load. Hence we need pedobarograph value to confirm this hypothesis. In addition we also found significant difference in hardness value between groups B and C in area 5, 7, and 8 because these areas are subjected to stress. Also there was no correlation of Shore index between right and left foot except there is negative correlation (correlation co-efficient r0−0.6) of shore index among foot area 5 between Group A with group B and group C as shown in Fig. 3. Therefore results appear to be keeping with other previous studies suggesting shore meters is a simple, non-invasive and cost effective device allowing determination of foot sole hardness which increases the understanding of the biomechanics and pathophysiology of the diabetic foot.

Clinical significance of our findings

In subjects with diabetic neuropathy, the shore value increases between 20 % to 35 % compared to subjects without diabetic neuropathy. This implies that shore value increase in the range of 30 to 40° shore predisposed towards the development of foot ulcers in areas with loss of protective sensation to 10gm SWMF. From the Tables 2 and 3, we infer that shore value is greater than 35° shore for the foot sole areas (heel, medial fore foot and big toe) which implies that the foot areas are at risk for ulcer formation. In addition, a combination of excess callosity outgrowing its blood supply causing central necrosis may lead to ulcer formation with continuous increase in plantar pressure. Finally it is important to remember that such foot assessment is important for prevention and early detection of diabetes combined with strict glycemic control in those diagnosed with diabetes.

Limitations

This study is not adequately powered, but the findings of this pilot study are very encouraging. Another limitation of this study is the inability to relate foot sole hardness to foot sole soft tissue thickness. A further limitation is that we did not have data on sweating as tested by the Neuropad® tester nor did we have data on neuropathy status using vibration perception threshold tester.

In conclusion of many methods suggested earlier for predicting foot ulceration in subject with diabetes mellitus but this study quantifies and shows the feasibility of measuring foot sole hardness using shore meter between three groups. With small numbers of subjects, our findings suggest that foot sole hardness is greater in subjects with diabetic neuropathy compared to subjects without diabetic neuropathy. The latter group has increased foot sole hardness compared to a control group. It is advisable to include persons without diabetes with neuropathic feature and compare their foot sole hardness with other groups. In addition, we need to establish reference values in the control population to see whether other factors such as gender, age, smoking, degree of exercise, and barefoot walking affect foot sole hardness. In addition we have to identify whether subjects with diabetic neuropathy differed from non-diabetic with neuropathy. Therefore an indentation principle tool might provide suitable guidelines to biomedical engineers and doctors to suggest orthotics devices for preventative management of foot ulcers in subject with diabetes mellitus.

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Conflict of interest All authors declare that they have no conflicts of interest.

References

1. Pecoraro RE, Reiber GE, Burgess EM. Causal pathways to amputation: basis for prevention. Diabetes Care. 1990;13:513–21.
2. Bell-Krotoski J, Weinstein S, Weinstein C. Testing sensibility, including touch-pressure, two point discrimination, point localization and vibration. J Hand Ther. 1993;6:114–23.
3. Weinstein S. Fifty years of somatosensory research: from the Semmes Weinstein monofilaments to the Weinstein enhanced sensory test. Hand Ther. 1993;6:11–28.
4. McIntosh A, Peters J, Young R, Hutchinson A, Chiverton R, Clarkson S, et al. Prevention and management of foot problems in type 2 diabetes: clinical guidelines and evidence. National Institute of Clinical Science; 2003.
5. World Health Organization. Guidelines for the prevention, management and care of diabetes mellitus. World Health Organization Geneva; 2006.
6. Feng Y, Schlösser FJ, Sumpio BE. The Semmes Weinstein monofilament examination as a screening tool for diabetic peripheral neuropathy. J Vasc Surg. 2009;50:675–82.
7. International Working Group on the Diabetic Foot. Practical guidelines of the diabetic foot. http://www.iwgdf.org/index.php?option0com_content&task0view&id031&Itemid024. Accessed 5 February 2011.
8. Piaggesi A, Romanelli M, Schipani E, Campi F, Magliaro A, Baccetti F, et al. Hardness of plantar skin in diabetic neuropathic feet. J Diabetes Complications. 1999;13:129–34.
9. Boulton AJM, Hardisty CA, Betts RP. Dynamic foot pressure and other studies as diagnostic and management aids in diabetic neuropathy. Diabetes Care. 1983;6:28–33.
10. Boulton AJM, Betts RP, Franks CL. Foot pressure studies in diabetic neuropathy. In: Proceedings of the International Conference on Biomechanics and Clinical Kinesiology of Hand and Foot, Chennai, India; 1985. p. 109–112
11. Patil KM, Srinath MS. New image processing system for analysis, display and measurement of static and dynamic foot pressures. Med Biol Eng Comput. 1990;28:416–22.
12. Veves A, Masson EA, Fernando DJ, Boulton AJM. Use of experimental padded hosiery to reduce abnormal foot pressures in diabetic neuropathy. Diabetes Care. 1989;12:653–5.
13. Patil KM, Babu M, Oommen PK, Malaviya GN. On-line system of measurement and analysis of standing and walking foot pressures

in normals and patients with neuropathic feet. Innov Technol Biol Med. 1996;17:401–8.
14. Cavanagh PR, Simoneau JS, Ulbrecht JS. Ulceration, unsteadiness, and uncertainty: the biomechanical consequences of diabetes mellitus. J Biomech. 1993;26:23–40.
15. Falanga V, Bucalo B. Use of durometer to assess skin hardness. Am J Dermatol. 1993;29:47–51.
16. Thomas VJ, Patil KM, Radhakrishnan S, Narayanamurthy VB, Parivalavan R. The role of skin, hardness, thickness and sensory loss on standing foot power in the development of plantar ulcers in patients with diabetes mellitus – a preliminary study. Int J Lower Extrem Wounds UK. 2003;2:132–9.
17. Charanya G, Patil KM, Narayanamurthy VB, Parivalavan R,
Visvanathan K. Effect of foot sole hardness, thickness and footwear on foot pressure distribution parameters in dia- betic neuropathy. Proc I MECH E Part H J Eng Med. 2004; H6:431–43.
18. Brink T. Induration of the diabetic foot pad: another risk factor for recurrent neuropathic plantar ulcers. Biomed Tech (Berl).
1995;40:205–9.
19. Thomas VJ, Patil KM, Radhakrishnan S. Three-dimensional stress analysis for the mechanics of plantar ulcers in diabetic neuropathy. Med Biol Eng Comput. 2004;42:230–5.
20. Aghassi D, Monoson T, Braverman I. Reproducible measurements to quantify cutaneous involvement in scleroderma. Arch Dermatol. 1995;13:1160–6.
21. Romanelli M, Falanga V. Use of a durometer to measure the degree of skin induration in lipodermatosclerosis. J Am Acad Dermatol. 1995;32:188–91.
22. LeBlanc N, Falabella A, Murata H, Hasan A, Weiss E, Falanga V. Durometer measurements of skin induration in venous disease. Dermatol Surg. 1997;23:285–7.
23. Merkel PA, Silliman NP, Denton CP, Furst DE, Khanna D, Emery P, et al. Validity, reliability, and feasibility of durometer measurements of scleroderma skin disease in a multicentre treatment trial. Arthritis Rheumatology. 2008;59:699–705.
24. Cavanagh PR, Rodgers MM, Liboshi A. Pressure distribution under symptom free feet during bare foot standing. Foot Ankle. 1987;7:262–76.
25. Kumar S, Fernando DJ, Veves A, Knowles EA, Young MJ, Boulton AJM. Semmes-Weinstein monofilaments: a simple, effective and inexpensive screening device for identifying diabetic patients at risk of foot ulceration. Diabetes Res Clin Pract. 1991;13:63–7.
26. Lee S, Kim H, Choi S, Park Y, Kim Y, Cho B. Clinical usefulness of the two-site Semmes-Weinstein monofilament test for detecting diabetic peripheral neuropathy. J Korean Med Sci. 2003;18:103–7.

ORIGINAL ARTICLE

Serum Adenosine deaminase activity in type 2 Diabetes Mellitus patients

M. N. Vanitha Gowda & K. C. Vasudha & S. Reshma & K. J. Sujatha

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Abstract The assessment of insulin resistance is advantageous since it detects those at risk for the development of diabetes mellitus at early stages and minimizes complications. Various methods like euglycemic hyperinsulinemic clamp technique, fasting serum insulin levels, HOMA and QUICKI have been used to determine insulin sensitivity but they have limitations. Adenosine deaminase (ADA) is an enzyme that has been suggested to be important for modulating the bioactivity of insulin. The aim of this study was to determine the activity of serum adenosine deaminase (ADA) in patients of type 2 diabetes mellitus and to evaluate the role of serum adenosine deaminase as a marker for insulin resistance. The study recruited 46 subjects of Type 2 diabetes mellitus and 40 healthy controls matched for age and sex between May 2010 and November 2010. Fasting serum glucose, insulin, adenosine deaminase activity were estimated. QUICKI and HOMA were calculated. Serum ADA was positively correlated with fasting serum glucose, insulin, HOMA and negatively correlated with QUICKI in the diabetic group. At 95 % CI and a cut off of 36.91U/L serum ADA activity in the diabetic group showed a sensitivity and specificity of 98 % and 90 % respectively. Serum ADA activity was increased with an increase in insulin resistance in the diabetic population. ADA may be used as a marker of insulin resistance and can be employed as an effective tool in screening for insulin resistance and diabetes mellitus.

Keywords Adenosine deaminase · Insulin resistance · Diabetes mellitus · HOMA · QUICKI

M. N. V. Gowda (*)
e-mail:**vanithasukesh@hotmail.com**

Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels. Autoimmune destruction of the β cells of the pancreas with consequent insulin deficiency and abnormalities that result in resistance to insulin action are the processes involved in the development of diabetes. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action [2].

Insulin resistance can be defined as the inability of insulin to produce its numerous actions (efficient utilisation of glucose by peripheral tissues especially muscle and liver), in spite of the unimpaired secretion from the beta cells [3]. Insulin resistance could be caused by various genetic and acquired conditions. Genetic causes for insulin resistance are rare and include antibodies against insulin receptor or mutations in the insulin receptor gene. Insulin resistance that is acquired results from impairments in cellular events distal to the interaction between insulin and its surface receptor [3].

Insulin is a peptide hormone produced by the pancreas in response to hyperglycemia and stimulates the utilization of glucose in various tissues (skeletal muscle, liver and adipose tissue). The net effect of the action of insulin on these tissues is to increase glucose uptake, reduce circulating glucose levels and increase the conversion of glucose into the storage molecules, glycogen or fat [4]. In addition to these classical insulin target tissues, there are many other important physiological targets of insulin, including the brain, pancreatic β cells, heart, and vascular endothelium, that help to coordinate and couple metabolic and cardiovascular

homeostasis under healthy conditions. In insulin resistance, adipose, muscle and liver cells do not respond appropriately to insulin, and circulating glucose levels remain high, which leads to complications. Insulin resistance is defined as decreased sensitivity or responsiveness to the metabolic actions of insulin [5]. Insulin resistance plays a major pathophysiological role in type 2 diabetes and is associated with obesity, hypertension, coronary artery disease, dyslipidemias and a cluster of metabolic and cardiovascular abnormalities that define the metabolic syndrome [6].

The assessment of insulin resistance is advantageous since it detects those at risk at an early stage and minimizes complications. The gold standard method for the determination of insulin sensitivity is the euglycemic hyperinsulinemic clamp technique which is both expensive and complex [7]. Hence, indices derived from one-off fasting specimen measurements (e.g. homeostasis model assessment and quantitative insulin sensitivity check index) are used. These suffer from limitations too [6, 7]. This necessitates the availability of a simple inexpensive marker with sufficient diagnostic efficiency.

Adenosine deaminase is a polymorphic enzyme that is ubiquitous in mammalian tissues. ADA catalyses the irreversible deamination of adenosine and 2′-deoxyadenosine to inosine and deoxyinosine in the purine catabolic pathway. Therefore, it regulates the intracellular and extracellular concentration of adenosine and 2′-deoxyadenosine molecules with many effects on human cells [8]. Humans have two known adenosine deaminase enzymes: ADA1 and ADA2. The function of ADA1 is to decrease intracellular levels of adenosine, an important signaling molecule, but one that is toxic at higher concentrations. In contrast, ADA2 is expressed extracellularly on the surface of many cells and has not only deaminase activity but also cytokine-like growth factor activity [9].

Adenosine deaminase (ADA) also plays an important role in lymphocyte maturation and activation [10]. Congenital deficiency of ADA causes severe combined immunodeficiency, which is characterized by absence of functional T and B lymphocytes. This condition has been attributed to the toxic effects of adenosine and deoxyadenosine that accumulate in the absence of ADA and impair lymphocyte differentiation and function [11]. Serum ADA activity has been found to be increased in several diseases where cellular immunity is stimulated such as liver diseases, tuberculosis, typhoid, infectious mononucleosis, tuberculosis, pneumonia, rheumatoid arthritis, lupus erythematosus, Acute Lymphoblastic Leukemia (ALL) and certain malignancies, especially those of hemopoietic origin [12, 13]. Studies suggest that serum levels of ADA rise in some diseases caused by microorganisms infecting mainly the macro- phages and in hypertensive disorders, which may represent a compensatory mechanism resulting from increased adenosine levels and the release of hormones and inflammatory mediators stimulated by hypoxia [12, 13]. ADA is also suggested to be an important enzyme for modulating the bioactivity of insulin [14–17]. This may be because ADA reduces the concentration of adenosine which in turn modulates the action of insulin on various tissues. Studies have correlated the increase in ADA activity with the degree of hyperglycemia [15]. Normalization of blood glucose level was associated with the decrease in ADA activity [18]. Studies correlating ADA activity to fasting insulin levels, HOMA and QUICKI have not been done. Since, ADA has been suggested to play a role in insulin effect and glycemic control, this study was undertaken to determine the activity of serum adenosine deaminase in patients of type 2 diabetes mellitus and to evaluate the role of serum adenosine deaminase activity as a marker for insulin resistance.

Materials and methods

Study pattern

A comparative case control study with 46 subjects of Type 2 diabetes mellitus and 40 healthy controls matched for age and sex was undertaken between May 2010 and November 2010. Ethical clearance was obtained from the institutional ethics committee. The subjects were selected based on the following inclusion and exclusion criteria.

Inclusion Criteria: Patients with diabetes mellitus (diagnosed not more than 6 years ago) in the age group 30–60 years, both sexes, either freshly diagnosed or on treatment with the oral anti-hyperglycemic agent metformin (biguanides).

Exclusion criteria: Subjects on insulin treatment, on drugs like sulfonylureas, thiazolidinediones, glucocorticoids, thyroid hormones, thiazides, diazoxides, pentamidine, phenytoin, a interferons or having a history suggestive of any infections, known complications of diabetes mellitus, liver disease, immunological disorders, trauma or malignancy.

Group 1: Cases: 46 adult diabetics attending the outpatient department of Medicine at MS Ramaiah Medical Teaching Hospital. This included 20 patients freshly diagnosed, not on treatment and 26 patients (who had been diagnosed less than 6 years ago) on treatment with the oral anti-hyperglycemic agent metformin. Diagnosis of type 2 diabetes mellitus was based on history and elevated fasting blood glucose.

Group 2: Controls: 40 healthy individuals as controls for the study were selected from the persons attending the outpatient department of Medicine at MS Ramaiah Medical Teaching Hospital for a routine health check-up.

Collection of samples

About 2 mL of blood was collected in a plain BD GEL Vacutainer tube after confirming that the subject has had no caloric intake for the past 8–12 h.

- Fasting serum glucose was done by Hexokinase method on Cobas 6000 c501 automated analyzer. A fasting serum glucose of ≤109 mg/dl was considered normal and values ≥126 mg/dl were considered diagnostic of diabetes mellitus [2].
- Fasting serum insulin was determined by ELISA method using monoclonal antibody based reagent (Bioline). Fasting insulin was considered to assess insulin resistance when fasting insulin levels ≥12 mU/l among both non-diabetic and diabetic populations [19]. Insulin resistance was assessed using Quantitative Insulin Sensitivity Check Index (QUICKI) and Homeostasis Model Assessment (HOMA). QUICKI 0 1/ $[\log(_{I0}) + \log(_{G0})]$, where I0 is fasting insulin (microunits per milliliter) and G0 is fasting glucose (milligrams per decilitre)[7]. HOMA [7] was calculated as follows

  HOMA ¼ Insulinðmicrounits=mLÞ

  glucoseðmmol=LÞ=22:5:x

  Subjects were considered as insulin resistant when HOMA ≥2.6 and QUICKI ≤0.33 [19].
- Serum Adenosine Deaminase (ADA) levels were estimated using a method reported by Giusti and Galanti [20].

Statistical analysis

Descriptive statistical analysis was carried out in the present study. Results on continuous measurements are presented on Mean ± SD (Min-Max) and results on categorical measurements are presented in Number (%). Significance was assessed at 5 % level of significance. Student t test (two tailed, independent) was used to find the significance of study parameters on continuous scale between two groups

Table 1 Comparison of bio-chemical parameters in two groups

Study variables	Controls	Cases	P value
Fasting blood glucose mg/dL	86.4±8.01	167.5±10.60	<0.001**
Adenosine deaminase U/L	19.26±9.38	48.01±1.26	<0.001**
Insulin μU/mL	6.52±2.25	16.95±3.04	<0.001**
QUICKI	0.36±0.03	0.29±0.02	<0.001**
HOMA	1.39±0.25	7.04±1.69	<0.001**

**Highly significant (P≤0.01)

Table 2 Comparison of bio-chemical parameters in freshly diagnosed patients of diabetes mellitus and in the patients on treatment

Study variables	Diabetics		P value
	Freshly diagnosed	On treatment	
Number of subjects	20	26	–
FBG (mg/dl)	208.70±46.01	147.03±27.07	<0.001**
Adenosine deaminase U/L	54.63±5.27	43.46±7.49	<0.001**
Insulin μUnits/mL	18.25±1.91	14.60±1.43	<0.001**
QUICKI	0.28±0.01	0.30±0.01	<0.001**
HOMA	9.54±2.97	5.36±1.41	<0.001**

+Borderline significance (P value: 0.05 < P < 0.10)

*Moderately significant (P value: 0.01 < P < 0.05)

**Highly significant (p value: p≤0.01)

Inter group analysis) on metric parameters. Pearson correlation between study variables was performed. Diagnostic statistics viz. Sensitivity, Specificity, and Area Under Curve was done by ROC curve analysis.

Results

Comparison of the biochemical parameters is shown in Table 1. Fasting glucose levels were normal in controls and significantly higher (P < 0.001) in diabetic subjects. The Adenosine deaminase levels were significantly (P < 0.001) higher in the diabetic group with an average value of 48.01 ± 1.26 U/L when compared to the control subjects (19.26 ± 9.38U/L). There were statistically significant differences (P < 0.001) between patients of diabetes mellitus and the controls in the fasting insulin levels, QUICKI and HOMA (Table 1).

There was a significant difference in fasting glucose values amongst the freshly diagnosed patients and the patients on treatment (Table 2). ADA, fasting insulin levels, QUICKI and HOMA between freshly diagnosed patients and the patients on treatment were also statistically significant (Table 2).

Table 3 Pearson correlation between ADA and insulin indices

Study variables	Controls		Cases	
	r value	P value	r value	P value
ADA(U/L) vs Fasting Blood Glucose	−0.124	0.392	0.773	<0.001**
ADA(U/L) vs Insulin μU/mL	0.052	0.718	0.701	<0.001**
ADA(U/L) vs QUICKI	0.051	0.726	−0.786	<0.001**
ADA(U/L) vs HOMA	0.035	0.810	0.762	<0.001**

Table 4 Comparison of ADA according to levels of insulin indices

Insulin Indices	Criteria	ADA (U/L)		P value
		Controls	Cases	
Insulin μU/mL	<12.0	19.04±9.21	–	–
	≥12.0	22.77±9.68	48.31±8.62	<0.001**
QUICKI	≤0.33	21.69±9.61	48.31±8.62	<0.001**
	>0.33	19.19±9.27	–	–
HOMA	<2.60	19.05±9.21	–	–
	<2.60	22.78±9.68	48.31±8.62	<0.001**

Table 3 Pearson's correlation coefficients between all the tested parameters and ADA levels. In the cases, ADA showed significantly strong correlation with FBG ($P < 0.001$), Insulin ($P < 0.001$), QUICKI ($P < 0.001$) and HOMA ($P < 0.001$). In the controls there was no linear correlation of ADA with FBG, Insulin, QUICKI or HOMA.

Table 4 shows that, at ADA levels of 48.31±8.62 U/L, the cut off levels of Insulin is ≥12.0μU/mL, QUICKI is≤ 0.33 and HOMA is ≥2.60 (Table 5).

Discussion

Type 2 DM is a heterogeneous disease characterized by altered protein, fat and carbohydrate metabolism secondary to insulin resistance. Identifying insulin resistance at an early stage helps in minimizing the complications. The current methods for measuring insulin resistance are not routinely used due to their complexity and limitations. Therefore, there is a need to identify a parameter whose estimation is simple, inexpensive and which can identify insulin resistance without actually requiring estimation of serum insulin.

The study intended to find the utility of ADA as a marker for insulin resistance and estimated the ADA levels and other indices of insulin resistance in Type 2 diabetes mellitus. The ADA levels were significantly elevated in diabetic group and also showed significant correlation with fasting blood glucose levels, fasting insulin levels, QUICKI and HOMA as shown in Fig. 1. The correlation was positive with fasting blood glucose levels, fasting insulin levels and HOMA, whereas negative with QUICKI. Other studies have also shown an increase in ADA levels in diabetic patients [15–17, 21] and a significant correlation with HbA1c [21] and BMI [22]. The mechanism that increases serum ADA activity in patients of diabetes mellitus is not well known. Adenosine deaminase is an enzyme that irreversibly deaminates adenosine to inosine, contributing to the regulation of intracellular and extracellular concentrations of adenosine. The increased ADA activity in hyperglycemic subjects would lead to a decrease in the level of adenosine. Adenosine increases glucose uptake into cells. A decrease in the level of adenosine contributes further to insulin resistance and associated cellular proliferation, inflammation and T-cell activity. ADA is therefore suggested to be an important enzyme for modulating the bioactivity of insulin [21, 23]. However, it is difficult to conclude whether changes in ADA activity are the cause or result of actual insulin resistance [24, 25]. The patients of diabetes mellitus in our study included 20 adults who were freshly diagnosed, not on treatment and 26 adults on treatment with the oral anti-hyperglycemic agent metformin (biguanides). The ADA levels were higher in newly diagnosed diabetics not on treatment (54.63±5.27 U/L) as compared to patients on treatment with metformin (43.46±7.49 U/L). Metformin is an effective hypoglycemic drug that lowers blood glucose concentrations by decreasing hepatic glucose production and increasing glucose disposal in skeletal muscle, mediated by the activation of AMP-activated protein kinase (AMPK) activity [26]. In a previous study, undertaken to determine whether ADA activity is affected by other therapeutic drugs, patients of type 2 diabetes mellitus on metformin monotherapy showed a lower ADA activity compared with that of those on sulfonylurea monotherapy [21]. This suggests the possibility that metformin can directly influence ADA activity. Also, metformin decreases insulin resistance, so ADA activity is expected to decrease in conjunction with metformin therapy [21].

Dipeptidyl-peptidase IV/CD26 (DPP IV) is a protease expressed on surfaces of T lymphocytes, endothelial and epithelial cells. It interacts with adenosine deaminase to bring about T cell activation and modulate chemotaxis [27]. It also plays a role in glucose homeostasis through proteolytic inactivation of the incretins. DPP IV inhibitors improve glucose tolerance and pancreatic islet cell function in animal models of type 2 diabetes and in diabetic patients. DPP IV is also implicated in HIV-1 entry, malignant transformation, and

Table 5 Comparison of insulin indices and ADA levels using ROC curve analysis

Study variables	Cut-off	Sensitivity	Specificity	LR+	LR-	AUC	95%CI
Adenosine Deaminase U/L	>36.91	98.00	90.00	10.00	0.0	0.981	0.94–0.99
Insulin μU/mL	>12.1	100.00	86.00	7.14	0.00	0.898	0.93–0.99
QUICKI	<0.32	97.83	92.00	12.23	0.02	0.976	0.82–0.95
HOMA	>3.59	100.00	92.00	12.50	0.00	0.979	0.92–0.99

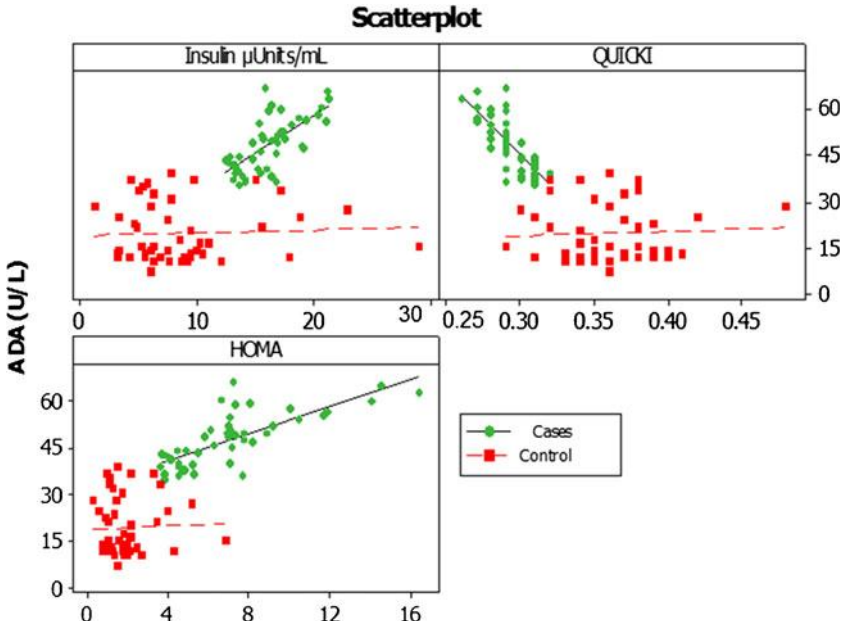

Fig. 1 Scatter plots for the correlation between ADA and other insulin indices

tumor invasion [28]. Other than binding to the glycoprotein CD26, ADA is also capable of binding to adenosine receptors A1 and A2A. These receptors are believed to play a role in regulating myocardial oxygen consumption, coronary blood flow, negative regulation of over-reactive immune cells, regulation of glutamate and dopamine release, making it a potential therapeutic target for the treatment of conditions such as insomnia, pain, depression, drug addiction and Parkinson's disease [29, 30]. Therefore further studies on ADA, its interaction with adenosine receptors and adenosine receptor antagonists may open new avenues with potential applications as drugs for treating cardiac, immune and other diseases.

The ADA levels also showed significant correlation with fasting insulin levels, HOMA and QUICKI. The correlation was positive with fasting insulin levels and HOMA, where as negative with QUICKI. Fasting insulin levels, HOMA and QUICKI are simple and established methods for the assessment of insulin resistance [7]. As shown in Fig. 2, at 95 % CI and a cut off of 36.91 U/L, ADA in the diabetic group showed sensitivity and specificity of 98 % and 90 % respectively. Upper limit of ADA in controls was 28.64 U/L, and this is significantly lesser than the lower cut off of 36.91 U/L in the type 2 diabetes group. Based on the area under ROC curve, a test is rated as excellent if its diagnostic value is between 0.9 and 1.0. Area under ROC curve for ADA showed its diagnostic value as 0.98 and hence, ADA may be used as a diagnostic tool for identifying insulin resistance. Also, it can be noted that ADA levels was higher in newly diagnosed diabetics (54.63 ± 5.27 U/L) as compared to patients on treatment (43.46 ± 7.49 U/L). Thus, the early onset of insulin resistance can be identified by elevated serum ADA activity and can be used during the follow up as it is low in treated patients.

This is a pilot study that has shown an increase in the ADA activity with an increase in insulin resistance in the diabetic population. This study may be extended to the pre-diabetic state (impaired fasting glucose and impaired glucose tolerance) with a larger number of samples. If in the latter, ADA levels were found to rise in correlation with the blood glucose of pre-diabetic range (Fasting serum glucose ≥110 mg/dl and <126 mg/dl and 2 h post-load glucose ≥140 mg/dl and <200 mg/dl [2]), it would be more

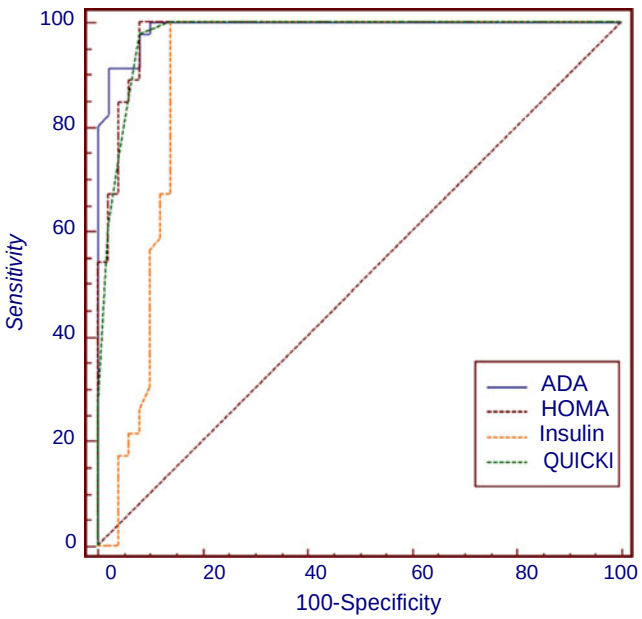

Fig. 2 shows ROC analysis for ADA, insulin, QUICKI and HOMA. ROC analysis for ADA showed a sensitivity of 98 % and a specificity of 90 % at a value >36.91 U/L. The figure shows area under ROC curve for ADA as 0.981

conclusive to accept serum ADA activity as an independent, inexpensive marker of insulin resistance.

A large number of studies have reported elevated serum ADA along with increased serum levels of AST, ALT and immunoglobulins in hepatitis [31],liver fibrosis and hepatoma [32]. These studies also suggest that serum ADA levels may be a new marker for liver disease, and should be considered a useful tool for the monitoring of liver condition especially in the treatment of patients with hepatitis [31]. We have not estimated serum transaminases and other organ function tests like serum creatinine in our study. In order to utilize Serum ADA levels for screening, studies in obese non-diabetics with insulin resistance and studies of organ function tests are required. The limitations of the study are that the transaminase levels which are known to be related to ADA, were not measured and a group of insulin resistance patients without diabetes were not included.

To conclude, ADA may be used as a marker of insulin resistance and can be employed as an effective tool in screening for insulin resistance and diabetes mellitus.

References

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2009;32 Suppl 1:S62–7.
2. The expert committee on the diagnosis and classification of diabetes mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 2003;26:S5–20.
3. Potenza MV, Mechanick JI. The metabolic syndrome: definition, global impact, and pathophysiology. Nutr Clin Pract. 2009;24:560–77.
4. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation. 2006;113:1888–904.
5. Huang PL. A comprehensive definition for metabolic syndrome. Dis Model Mech. 2009;2:231–7.
6. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. Am J Physiol Endocrinol Metab. 2008;294:15–26.
7. Borai A, Livingstone C, Ferns GAA. The biochemical assess- ment of insulin resistance. Ann Clin Biochem. 2007;44:324–42.
8. Edwards YH, Hopkanson DA, Hams H. Adenosme deaminase lsozymes in human tissues. Ann Hum Genet. 1971;35:207–18.
9. Van der Weyden MB, Kelly WN. Human adenosine deaminase: distribution and properties. J Biol Chem. 1976;251:5448–56.
10. Blackburn MR, Kellems RE. Adenosine deaminase deficiency: metabolic basis of immune deficiency and pulmonary inflammation. Adv Immunol. 2005;86:1–41.
11. Baghanha MF, Pego A, Lima MA, et al. Serum and pleural adenosine deaminase correlation with lymphocyte populations. Chest. 1990;87:605–10.
12. Ungerer JP, Oosthuizen HM, Bissbort SH, Vermaak WJ. Serum adenosine deaminase: isoenzymes and diagnostic application. Clin Chem. 1992;38:1322–6.
13. Matteucci E, Giampietro O. Dipeptidyl peptidase-4 (CD26): knowing the function before inhibiting the enzyme. Curr Med Chem. 2009;16:2943–51.
14. Kurtul N, Pence S, Akarsu E, et al. Adenosine deaminase activity in the serum of type 2 diabetic patients. Acta Medica(Hradec Kralove). 2004;47:33–5.
15. Hoshino T, Yamada K, Masuoka K, et al. Elevated adenosine deaminase activity in the serum of patients with diabetes mellitus. Diabetes Res Clin Pract. 1994;25:97–102.
16. Warrier A, Rao NY, et al. Evaluation of Adenosine Deaminase activity and lipid peroxidation levels in Diabetes Mellitus. IJCB. 1995;10:9–13.
17. Rutkiewicz J, Gorski J. On the role of insulin in regulation of adenosine deaminase activity in rat tissues. FEBS Lett. 1990;271:79–80.
18. McAuley KA, Williams SM, Mann JI, Walker RJ, Lewis-Barned NJ, Temple LA, et al. Diagnosing insulin resistance in the general population. Diabetes Care. 2001;24:460–4.
19. Gupta A, Gupta V, Agrawal S, Natu SM, Agrawal CG, Negi MPS, Tiwari S. Association between circulating leptin and insulin resistance, the lipid profile, and metabolic risk factors in North Indian adult women. BioScience Trends. 2010;4:325–32.
20. Giusti G, Galanti B. Colorimetric method. Adenosine deaminase. In: Bergmeyer HU, editor. Methods of enzymatic Analysis. 3rd ed. Weinheim: Verlag chemie; 1984. p. 315–23.
21. Lee JG, Kang DG, Yu JR, Kim Y, Kim J, Koh G, et al. Changes in adenosine deaminase activity in patients with type 2 diabetes mellitus and effect of DPP-4 inhibitor treatment on ADA activity. Diabetes Metab J. 2011;35:149–58.
22. Bottini E, Gloria-Bottini F. Adenosine deaminase and body mass index in non-insulin-dependent diabetes mellitus. Metabolism. 1999;48:949–51.
23. Bopp A, De Bona KS, Bellé LP, Moresco RN, Moretto MB. Syzygium cumini inhibits adenosine deaminase activity and reduces glucose levels in hyperglycemic patients. Fundam Clin Pharmacol. 2009;23:501–7.
24. Koopmans SJ, Sips HC, Bosman J, Radder JK, Krans HM. Antilipolytic action of insulin in adipocytes from starved and diabetic rats during adenosine-controlled incubations. Endocrinology. 1989;125:3044–50.
25. Green A, Newsholme EA. Sensitivity of glucose uptake and lipolysis of white adipocytes of the rat to insulin and effects of some metabolites. Biochem J. 1979;180:365–70.
26. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes. 2002;51:2074–81.
27. Pérez-Aguilar MC, Goncalves L, Ibarra A, Bonfante-Cabarcas R. Adenosine deaminase as costimulatory molecule and marker of cellular immunity. Investig Clin. 2010;51:561–71.
28. Lambeir AM, Durinx C, Scharpé S, De Meester I, Dipeptidyl-peptidase IV. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Sciences. 2003;40:209–94.
29. Gracia E, Pérez-Capote K, Moreno E, Barkešová J, Mallol J, Lluís C, et al. A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. Biochem J. 2011;435:701–9.
30. Samsel M, Dzierzbicka K. Therapeutic potential of adenosine analogues and conjugates. Pharmacol Rep Online. 2011;63:601–17.
31. Kaya S, Cetin ES, Aridogan BC, Arikan S, Demirci M. Adenosine deaminase activity in serum of patients with hepatitis—a useful tool in monitoring clinical status. J Microbiol Immunol Infect. 2007;40:288–92.
32. Kobayashi F, Ikeda T, Marumo F, Sato C. Adenosine deaminase isoenzymes in liver disease. Am J Gastroenterol. 1993;88:266–71.

GUEST EDITORIAL

Diabetic nephropathy: associated risk factors in renal deterioration

Dhruv K. Singh

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Diabetic Nephropathy (DN) is now recognised as the leading cause of end stage renal disease (ESRD) and contributes between 20 and 40 % of patients with chronic kidney disease on renal replacement therapy (dialysis), worldwide [1]. Clinically, microalbuminuria is considered as the earliest sign of evolving DN, which may become persistent and progressive, leading to proteinuria due to combination of several factors such as chronic hyperglycemia, uncontrolled hypertension, ethnicity, family history of renal disease, lack of early intervention and/or suboptimal glycemic control [2].

Established DN is defined as presence of persistent proteinuria of >0.5 gm/day, hypertension and a progressive decline in the glomerular filtration rate (GFR), leading to ESRD [3]. Traditionally, the initiation and progression of diabetic nephropathy has been described to go through five different stages such as glomerular hyperfiltration, incipient nephropathy, microalbuminuria, overt proteinuria and end-stage renal disease [4]. In the Indian diabetes population, the prevalence of persistent microalbuminuria and DN has been reported to be approximately 27 and 2.2 %, respectively [5].

In subjects with type 1 diabetes, proteinuria has been reported to manifest in about 15–40 % of patients, and is generally seen in those with history of about 15–20 years of diabetes duration [6]. The prevalence of proteinuria in subjects with type 2 diabetes is highly variable with reported incidence ranging between 5 and 20 % in different populations [7]. The onset of proteinuria is associated with a significantly progressive decline in the GFR, with an average drop of approximately 10–12 ml/min/year [8]. There is a significant inter-individual difference in the rate of deterioration in type 1 and type 2 diabetes, however, the collective risk of progression to ESRD after 5 years of persistent proteinuria is approximately 60 % in both type 1 and type 2 diabetes [9].

In view of the increasing number of diabetes subjects progressing to ESRD, it is vital to identify the risk factors for deterioration of renal function to develop appropriate intervention therapies, to arrest the progression of DN. Perhaps, due to different etiopathogenesis, type 1 and type 2 diabetes subjects with nephropathy have been reported to have differential inter-individual rate of decline in renal function, as reflected by GFR [3, 10]. Although, progressive proteinuria is known to be the single most important factor for decline of renal function in DN, there are several other associated risk factors, which may significantly increase the risk of renal deterioration.

In type 1 diabetes subjects, several risk factors such as high normal urinary albumin excretion rate, male gender, hypertension, glycemic control (HbA1c), small height at baseline contribute to the evolution of persistent microalbuminuria [6]. In type 1 diabetes subjects with established DN, hypertension, albuminuria, hyperglycemia and hypercholesterolemia have been reported to promote the deterioration of renal function [3].

In type 2 diabetes subjects, baseline level of albuminuria, systolic blood pressure, glycemic control (HbA1c), GFR, age, heavy smoking, anemia and grade of retinopathy (none/background, proliferative), have been reported to be significant predictors for progressive renal failure as determined by rate of loss of GFR and time to doubling of baseline serum creatinine [11]. Surprisingly, gender, diabetes duration, diastolic blood pressure, body mass index and lipid levels did not show any significant association with progressive loss of GFR in this study [11].

Although baseline albuminuria and GFR are significant predictors of progressive renal deterioration, baseline albuminuria has been reported to have greater odds ratio for

D. K. Singh (✉)
drdksingh1@hotmail.com

deterioration of renal function, as compared to baseline GFR [12]. About 40–55 % of type 2 diabetes subjects have been reported to have reduced GFR in absence of microalbuminuria/proteinuria [13, 14]. This may be due to complex etiopathogenesis of type 2 diabetes and is also reflected in the histological presentation with only 30 % having classical findings of thickening of glomerular basement membrane and mesangial expansion, the other 30 % with normal renal structure and the remaining 40 % having predominant tubulointerstitial lesions such as tubular atrophy and tubular basement membrane thickening with relatively mild glomerular changes [15].

In a subset analysis of UKPDS study on type 2 diabetes subjects to determine the factors for decline in renal function, a diverse set of predictors were identified for progression of albuminuria and progressive decline in GFR [16], suggesting an interplay of complex mechanisms in the manifestation of both these pathological processes. Persistent microalbuminuria has been considered to be the most important risk factor for development of proteinuria, however, not all patients with microalbuminuria progress to overt proteinuria neither do all patients with deteriorating GFR have preceding microalbuminuria/proteinuria [16], which is intriguing.

Clinically, presence of persistent microalbuminuria in diabetes subjects warrants intervention therapies to prevent its progression. With the introduction of angiotensin converting enzyme inhibitors (ACE) and angiotensin receptor blockers (ARB) group of anti-hypertensives, the rate of progression from microalbuminuria to proteinuria in type 1 diabetes subjects has seen a significant drop from as high as approximately 80 % [17] in the eighties to 44 % in the last decade [18]. However, a significant number of diabetes subjects continue to have progressive disease in spite of the above therapies, suggesting presence of other factors such as oxidative stress and/or genetic predisposition. Unfortunately, the above mentioned studies [3, 6, 11, 16] on progression of DN in type 1 and type 2 diabetes subjects were not designed to examine the contribution of oxidative stress or any genetic factors.

Ongoing oxidative stress in the glomerulus and tubules, as a result of chronic hyperglycemia has been proposed to be a major culprit in the initiation and progression of diabetic renal disease [19] and containment of oxidative stress has been shown to delay the progressive decline in GFR [20]. Chronic hyperglycemia promotes oxidative stress injury directly through processes such as enhanced production of advanced glycation end products (AGEs), enhanced reactive oxygen species (radical molecules such as superoxide, hydroxyl, peroxyl and non–radical molecules such as hydrogen peroxide and hydrochlorous acid) generation, reduced production of antioxidants such as nitric oxide, increased cytokine activity, enhanced inflammatory markers and endothelial dysfunction [1]. In addition, chronic hyperglycemia modulates several metabolic pathways such as oxidative phosphorylation, sorbitol/aldose reductase pathway or the polyol pathway, mitochondrial electron transport chain (ETC) and NAD(P)H oxidase [21] leading to enhanced oxidative stress burden with increased ROS formation and reduced NO production.

In conjunction with chronic hyperglycemia, oxidative stress may orchestrate several functional and structural abnormalities in the glomerulus and tubulointerstitium such as enhanced deposition of extracellular matrix in the mesangium, promotion of a hypoxic environment in the tubulointerstitium, enhanced oxidant injury and tubular apoptosis leading to tubulointerstitial fibrosis even before the onset of microalbuminuria [22], a state of generalised endothelial dysfunction [6], which may further aggravate the condition. Ongoing cellular oxidative stress has been postulated to be a significant factor in the progression of renal diseases [20]. With chronic hyperglycemia as a permanent feature, it is plausible that irrespective of microalbuminuria/proteinuria, oxidative stress plays a vital role in the progression of DN and contributes to renal deterioration [23].

Although it seems an exciting proposition, the confirmatory/contributory role of oxidative stress in the pathogenesis and progression of DN is yet to be established. Normally surrogate markers of oxidative stress, such as measurement of capacity of the vascular endothelial cells to release nitric oxide in response to ischemic stimuli is tested to determine the anti-oxidant response [24]. This is primarily due to lack of standardised tools to measure and quantify oxidative stress in chronic diseases including DN. The measurement of ROS in serum is hampered due to highly reactive nature of these molecules. Research studies in estimation of oxidative stress currently measure the total antioxidant buffering capacity of plasma or specific markers of free radical-mediated damage such as F(2)-isoprostane or oxidised-LDL (Ox-LDL) [25]. However, the robustness and consistency of these methods are yet to be validated in big populations and they largely remain research tools at the moment — far away from their application in a clinical setting.

In conclusion, the initiation and progression of DN is a complex process with varied contribution of several diverse metabolic and pathogenic processes in the background of chronic hyperglycemia. Baseline hypertension, albuminuria, glycemic control and dyslipidemia are common determinants of progressive decline in renal function in both type 1 and type 2 diabetes subjects with established DN, whereas, low GFR at baseline, increasing, age, heavy smoking, anemia and grade of retinopathy are additional factors associated with progressive decline in renal function in type 2 diabetes subjects. In addition, to the above mentioned

contributing factors, ongoing oxidative stress is associated with progressive renal disease [20].

A sizable portion of diabetes subjects do not develop microalbuminuria in spite of chronic hyperglycemia suggesting presence of certain yet unidentified protective factors (genetic or others). In addition, a progressive decline in GFR is also seen in a significant proportion of diabetes subjects without microalbuminuria/proteinuria, suggesting that decline in renal function is not dependent on presence of proteinuria. Hence it is desirable that all diabetes subjects should have measurement of GFR at least annually, along with microalbuminuria/proteinuria to monitor the renal function in general and identification of high risk DN patients who may have significantly greater rate of decline in GFR. Oxidative stress as a result of chronic hyperglycemia appears to be a major contributor to progressive decline in GFR in diabetes subjects and currently lot of research is underway to develop reliable tools to measure and monitor oxidative stress in these subjects.

The treatment cost of DN and its potential consequence such as ESRD is expensive and may not be affordable to a vast majority of the burgeoning population of patients with diabetes in the developing countries. Until specific therapies aimed at arresting progression of renal decline are available, optimum management of hyperglycemia, hypertension, dyslipidemia, lifestyle modification (cessation of smoking, regular exercise, stress free life and balanced diet) and regular monitoring of renal parameters (serum creatinine, albuminuria and GFR) with timely intervention, if required, remains the cornerstone of management of DN in diabetes subjects.

References

1. Hakim FA, Pflueger A. Role of oxidative stress in diabetic kidney disease. Med Sci Monit. 2010;16:RA37–48.
2. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. N Engl J Med. 1984;310:356–60.
3. Hovind P, Rossing P, Tarnow L, Smidt UM, Parving HH. Progression of diabetic nephropathy. Kidney Int. 2001;59:702–9.
4. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient dia- betic nephropathy. Diabetes. 1983;32 Suppl 2:64–78.
5. Unnikrishnan RI, Rema M, Pradeepa R, Deepa M, Shanthirani CS, Deepa R, et al. Prevalence and risk factors of diabetic nephropathy in an urban South Indian population: the Chennai Urban Rural Epidemiology Study (CURES 45). Diabetes Care. 2007;30:2019–24.
6. Hovind P, Tarnow L, Rossing P, Jensen BR, Graae M, Torp I, et al. Predictors for the development of microalbuminuria and macro- albuminuria in patients with type 1 diabetes: inception cohort study. BMJ. 2004;328:1105.
7. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int. 2003;63:225–32.
8. Remuzzi G, Schieppati A, Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. N Engl J Med. 2002;346:1145–51.
9. Hasslacher C, Ritz E, Wahl P, Michael C. Similar risks of nephropathy in patients with type I or type II diabetes mellitus. Nephrol Dial Transplant. 1989;4:859–63.
10. Nosadini R, Velussi M, Brocco E, Bruseghin M, Abaterusso C, Saller A, et al. Course of renal function in type 2 diabetic patients with abnormalities of albumin excretion rate. Diabetes. 2000;49:476–84.
11. Rossing K, Christensen PK, Hovind P, Tarnow L, Rossing P, Parving HH. Progression of nephropathy in type 2 diabetic patients. Kidney Int. 2004;66:1596–605.
12. Meguro S, Shigihara T, Kabeya Y, Tomita M, Atsumi Y. Increased risk of renal deterioration associated with low e-GFR in type 2 diabetes mellitus only in albuminuric subjects. Intern Med. 2009;48:657–63.
13. MacIsaac RJ, Tsalamandris C, Panagiotopoulos S, Smith TJ, McNeil KJ, Jerums G. Nonalbuminuric renal insufficiency in type 2 diabetes. Diabetes Care. 2004;27:195–200.
14. Thomas MC, MacIsaac RJ, Jerums G, Weekes A, Moran J, Shaw JE, et al. Nonalbuminuric renal impairment in type 2 diabetic patients and in the general population (national evaluation of the frequency of renal impairment cO-existing with NIDDM [NEFRON] 11). Diabetes Care. 2009;32:1497–502.
15. Dalla VM, Saller A, Bortoloso E, Mauer M, Fioretto P. Structural involvement in type 1 and type 2 diabetic nephropathy. Diabetes Metab. 2000;26 Suppl 4:8–14.
16. Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR. Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. Diabetes. 2006;55:1832–9.
17. Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med. 1984;311:89–93.
18. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. N Engl J Med. 2003;348:2285–93.
19. Singh DK, Winocour P, Farrington K. Oxidative stress in early diabetic nephropathy: fueling the fire. Nat Rev Endocrinol. 2011;7:176–84.
20. Goicoechea M, de Vinuesa SG, Verdalles U, Ruiz-Caro C, Ampuero J, Rincon A, et al. Effect of allopurinol in chronic kidney disease progression and cardiovascular risk. Clin J Am Soc Nephrol. 2010;5:1388–93.
21. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, et al. NADPH oxidases in cardiovascular health and disease. Antioxid Redox Signal. 2006;8:691–728.
22. Bagby SP. Diabetic nephropathy and proximal tubule ROS: challenging our glomerulocentricity. Kidney Int. 2007;71:1199–202.
23. Sandesh M, Kiran K, Jyoti M. Diabetic nephropathy and associated risk factors for renal deterioration. Int J Diab Dev Countries. 2012;32:52–59.
24. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial ar- tery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol. 2002;39:257–65.
25. Stephens JW, Khanolkar MP, Bain SC. The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease. Atherosclerosis. 2009;202:321–9.