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Research Note

Does the absolute HbA_{1c} improve the genotype-phenotype association in Type 2 Diabetes?

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Abstract. HbA_{1c} is a measure of the mean blood glucose levels for the prior 90 - 120 days, the mean lifetime of red blood cells. However, factors that influence the erythrocyte turnover or the biochemical structure of haemoglobin (Hb) might complicate the interpretation of results. With a frequency of haemoglobinopathies of around 5% in the Maltese population, an alternative biomarker should be considered. The aim of this study was to determine whether the absolute HbA_{1c} could improve the genotype-phenotype association in Type 2 Diabetes Mellitus (T2DM) and whether it could thus be an alternative measure.

Ion-exchange high performance liquid chromatography (HPLC) and polymerase chain reaction (PCR) were used to genotype and phenotype five different groups of subjects: haematologically normal adult controls, anaemics (Hb<10g/dL), β-thalassaemics, normal pregnant women and type 2 diabetics (controlling their diabetes either by diet alone, or using metformin for up to six months). The single nucleotide polymorphisms (SNPs) selected were in the ADRB2, LEP, FABP2, TCF7L2, MIF, IL6 and UCP1 genes.

Statistical analysis showed that the absolute HbA_{1c} did not improve the genotype-phenotype association, as it showed the same trends as the relative HbA_{1c}. The difference between the HbF and HbA_{1c} is due to the homogenous distribution of HbA_{1c} among erythrocytes, unlike HbF. *In vitro* glycation showed that Hb Beta-Valletta, found in 1.8% of Maltese adults, does not influence glycation and thus the HbA_{1c} is not influenced by this variant in heterozygotes/ homozygotes.

Keywords HbA_{1c} - Hb variants - HPLC - diabetes - SNPs - genetics.

Hb: haemoglobin
T2DM: Type II Diabetes mellitus
HPLC: High-performance liquid chromatography; RP-HPLC: Reverse phase- HPLC
Polymerase chain reaction
SNPs: Single nucleotide polymorphisms
DM: Diabetes mellitus
WHO: World Health Organisation
PBS: Phosphate-buffered saline
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1 Introduction

Diabetes mellitus (DM) is the most common serious metabolic disorder worldwide (Berg et al., 2006). According to WHO , around 2.8% of the global population currently suffer from type 2 diabetes mellitus (T2DM) and this value is expected to rise to around 4.4% in 2030 (Sicree and Shaw, 2007).T2DM is a complex disorder that may be considered an intermediate step in the progression from glucose intolerance to pre-prandial hyperglycaemia and macro- and micro-vascular complications (American Diabetes Association, 2012; Bennett et al., 2007). It is a heterogeneous disorder that is influenced by both genetic and environmental/ lifestyle factors, such as obesity and age (Olefsky, 2001).

The need for testing is important as preventative measures may decrease the risk of developing T2DM, or in the least retard the development of cardiometabolic complications (Diabetes Control and Complications Trial Research Group, 1993). The HbA_{1c}, a measure of the mean glucose concentration over the lifetime of the red blood cell (90-120 days on average) (Bennett et al., 2007), is commonly used to diagnose T2DM. Its suitability stems from its independence from prandial status, diurnal fluctuations and exercise (Sacks, 2003).

HbA_{1c} is formed by the process of non-enzymatic glycation: condensation of glucose to the free amine group of the N-terminal valine on the β-chain followed by the Amadori rearrangement (Bunn et al., 1976; Peterson et al., 1998; Saudek et al., 2006; Schulz, 2006; Sicree and Shaw, 2007). The accuracy of the HbA_{1c} may be influenced by factors that have an effect on the structure and half-life of red blood cells (Bernstein, 1980; de Boer et al., 1980; Eberentz-Lhomme et al., 1984; Fluckiger et al., 1981; Hanson et al., 1983; Horton and Huisman, 1965; Huisman et al., 1983; Lind and Cheyne, 1979; Paisey et al., 1986; Phelps et al., 1983; Starkman et al., 1983; Tran et al., 2004). With a 2% prevalence of thalassaemia and around 5% prevalence of haemoglobinopathies and red blood cell disorders in the Maltese Islands (Felice, 2012), it is important to consider other indicators of hyperglycaemia, such as the absolute HbA_{1c}, that may be calculated simply and non-invasively or quantified directly by immunoassay or mass spectrometry. This was the objective of this study.

2 Methods

Ethics approval for this study was obtained from the University Research Ethics Committee of the University of Malta.

Phenotyping: The HbA_{1c}, HbA_2 and HbF of 62 haematologically normal adults (N), 95 haematologically normal pregnant women (P), 61 β-thalassaemics (T), 39 severe anaemics (A) and 100 type 2 diabetics (D) (on limited or no treatment) were determined using ion-exchange HPLC (Bio-Rad Beta-thalassaemia short program kit on Bio-Rad VARIANT) within one week of whole blood collection. The haemoglobin concentration was obtained from the CBC. The absolute HbA_{1c} was calculated as follows (Sinha et al., 2012):

$$\text{Absolute Hb } A_{1c} = \frac{\text{HbA}_{1c}(\%) \times [Hb]}{100}$$

Genotyping: DNA from the aforementioned samples together with 200 random neonates (C), was extracted using a salting out procedure. PCR (simple, allele-specific and tetra-arms PCR) was used to genotype the following SNPS:rs1042713 (ADRB2), rs7799039 (LEP), rs1799883 (FABP2), rs7903146 (TCF7L2), rs755622 (MIF), rs603573 (IL6), rs1800592 (UCP1) (Abou-Hussein, 2009; Al Ashtar, 2008).

In vitro glycation: 5 normal samples and 3 blood samples from Hb Beta-Valletta heterozygotes were incubated with varying concentrations of glucose (0mM to 100mM in 10mM increments) in phosphate-buffered saline (PBS). The mixtures were incubated at 37°C for two hours prior to analysis using ion-exchange HPLC (Bio-Rad Beta-thalassaemia short program kit on Bio-Rad VARIANT).

In another experiment, 2 normal adult blood samples were incubated with 0mM, 50mM and 100mM glucose in PBS solutions for two hours at 37°C in a water bath. These were then analysed by reverse phase (RP)HPLC (Bio-Rad VARIANT with external JASCO UV-975 UV/Vis detector set at 215nm).

Data Analysis: Descriptive analysis, One Way Anova, scatter diagrams and correlations were carried out using IBM SPSS v17.0.Allele frequencies were calculated as follows:

$$p = f(AA) + \frac{1}{2} f(Aa)$$

$$q = f(aa) + \frac{1}{2} f(Aa) = 1 - p$$

3 Results

Phenotyping: As seen in table 1, diabetics and pregnant women exhibited the greatest and lowest values respectively, with normal adults closely following the latter. Thalassaemics and anaemics exhibited intermediary values with anaemics giving somewhat higher values.

Table 1: Values of glycation.

		HbA_{1c} (%)	Abs HbA_{1c}(g/dL)	HbA_{1c}/total HbA	Hb A_{1c}/(HbA_0 + HbA_{1c})
N	Avg.	4.88	0.66	5.06	5.31
	Std. Dev.	0.50	0.10	0.83	0.55
P	Avg..	4.38	0.53	4.62	4.77
	Std. Dev.	0.42	0.11	0.45	0.47
T	Avg.	5.54	0.61	5.99	6.29
	Std. Dev.	1.19	0.22	1.37	1.42
A	Avg.	6.12	0.64	6.44	6.62
	Std. Dev.	1.74	0.23	1.85	1.88
D	Avg.	8.14	1.14	/	/
	Std. Dev.	3.56	0.54	/	/

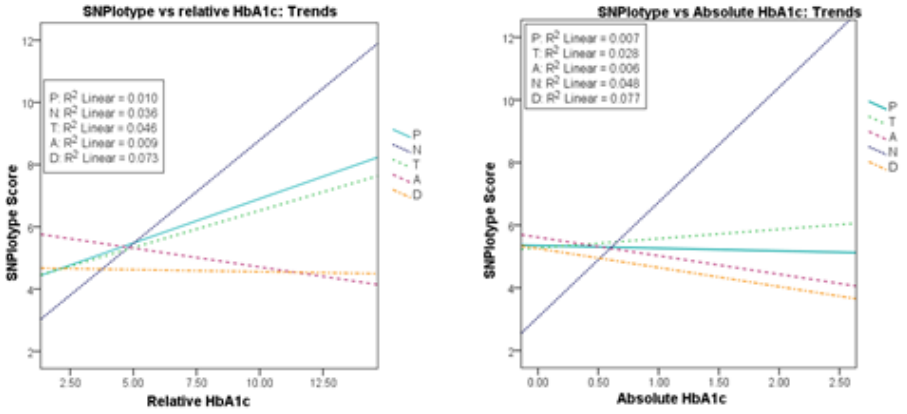

Figure 1: SNPlotype plots for the trends of the relative HbA_{1c} and the absolute HbA_{1c}.

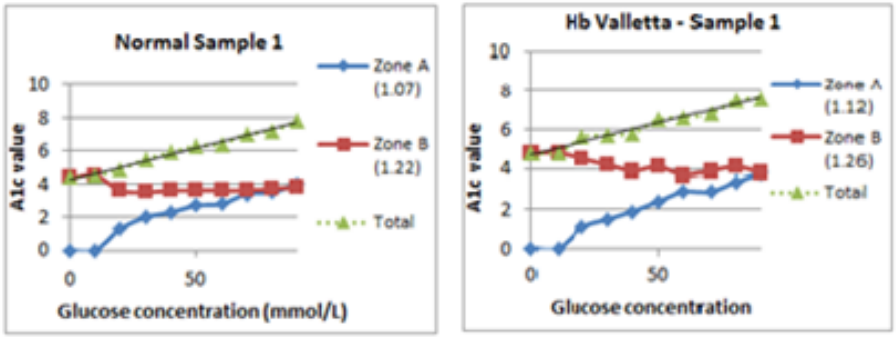

Figure 2: Overview of *in vitro* glycation results.

Diabetics exhibited a mean significant difference compared to all the other groups for both the relative and absolute HbA_{1c} at the 0.05 level and was the only significantly different group when considering the mean absolute HbA_{1c}.

SNPlotypes: The trend noted for normal adults (Fig. 1) was a proportional increase in glycation and genetic risk (as denoted by an increase in SNPlotype). This trend was not seen for the other groups. An inversely proportional relationship was observed for diabetics (Fig. 2).

Allele frequencies and correlations: These varied from one group to the next, with significant differences shown between cords and normal adults. Significant correlations were noted for the HbA_{1c} with MIF in pregnant women and ADRB2 in diabetics. No significant correlations were observed for the absolute HbA_{1c}.

Invitroglycation: On increasing the glucose concentration for both normal adult and Hb Beta-Valletta samples, the zone representing the HbA_{1c} (zone B in Fig. 2) was seen to increase in area. When the glucose concentration was increased above normal physiological conditions, zone A was eluted and increased with increasing concentrations. The appearance of this zone was accompanied by an initial decrease in the HbA_{1c} percentage. RP-HPLC indicated that this new zone was not the result of modification to the α- and β-globin structures.

4 Discussion

The absolute HbA_{1c} enabled a better distinction between the different groups based on phenotype alone but it did not offer an improved association with the genotype. Glucose is circulated uniformly throughout the blood resulting in equal exposure of all haemoglobin to extracellular glucose. HbA_{1c} is distributed evenly throughout the blood since it is not influenced by selective survival. The absolute HbA_{1c} does not improve the genotype-phenotype association and both the relative and absolute HbA_{1c} are equally useful measures of glycation.

The different trends obtained indicate that the hyperglycaemic state noted in diabetics was not attributed to genetic risk but to lifestyle choices such as obesity and poor diet. In fact, a significant proportion of Maltese individuals are known to be overweight or obese (Savona-Ventura, 2001).

The allele frequencies show the importance of choosing the correct control population. Random cords represent the general population while the normal adults represent

individuals with normal levels of glycation and normal haemoglobin physiology. Thus, both types of controls were needed for this study.

Haemoglobin variants, such as Hb Marseille, influence the measurement of glycated haemoglobin. Incubation with glucose indicated that, although no hyperglycaemic Hb Beta-Valletta individuals were encountered in the laboratory, this variant does not influence the HbA_{1c} value.

The elution of Zone A may indicate that non-enzymatic glycation occurred at more than one site. Apart from the N-terminal valine, five lysine residues are available for glycation on the β-globin subunit. These are not glycated in HbA_{1c}. The earlier elution time of Zone A, indicating a greater negative overall charge, and the decrease of HbA_{1c} with increasing abundance of the new zone suggest that the positively charged lysine residues are undergoing non-enzymatic glycation.

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