Hemoglobin A_{1c} Can Be Analyzed in Blood Kept Frozen at −80°C and Is Not Commonly Affected by Hemolysis in the General Population

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The ability of glycated hemoglobin A_{1c} (HbA_{1c}) to predict diabetes is unknown, but could be evaluated by analyses on samples stored in biobanks. The stability of HbA_{1c} in long-term stored samples is, however, unknown. Moreover, the effect of hemolysis on HbA_{1c} in the general population is not assessed. To explore these questions HbA_{1c} was determined in 3 groups (n = 717) of samples with storage times at −80°C differing between 10 years and 2 months. The results were compared with HbA_{1c} analyzed in fresh blood samples (n = 174). The subjects were free from diabetes and aged 40 to 60 years. HbA_{1c} was analyzed by cation exchange high-performance liquid chromatography (HPLC). The mean HbA_{1c} results for the fresh and long-term stored samples were 4.25% ± 0.39 and 4.19% ± 0.43, respectively (*P* = not significant [NS]). The HbA_{1c} levels in fresh and 2-month stored samples were essentially equal. There was no correlation between HbA_{1c} in the fresh samples and the hemolysis parameters reticulocytes, haptoglobin, or bilirubin. HbA_{1c} is apparently stable in samples long-term stored at −80°C and is not commonly affected by hemolysis in the general population. HbA_{1c} analyzed on biobank samples could be used to assess the predictive value for future diabetes and relationship to other morbidity and mortality.

DIABETES MELLITUS is a chronic disease that is classified into 2 major subgroups, type 1 and type 2 diabetes. Increased blood glucose associated with the risk of developing microvascular complications[1,2] defines both diabetes types. The recommended method for diagnosing diabetes mellitus is repeated measurements of fasting plasma glucose according to the World Health Organization (WHO) latest revised recommendation.[3] A previously recommended method has been the oral glucose tolerance test (OGTT), a method that has been de-emphasized in the revised WHO recommendation due to its variability,[4] and because it is time consuming for both patients and personnel.

In recent years, it has been suggested that measurement of the glycated fraction A_{1c} of hemoglobin (HbA_{1c})[5-8] could be an alternative to the methods above in the diagnosis of diabetes mellitus. HbA_{1c} has to date not been accepted as a diagnostic tool mainly due to difficulties in standardizing different analyzing methods. There are also some case reports indicating that hemolysis can be a cause of low HbA_{1c} values.[9,10] These studies have not been performed in the general population. Moreover, it has not been fully evaluated if HbA_{1c} can predict future diabetes. This question could be addressed using deep freeze stored blood samples that are available in medical biobanks.

We performed this study to evaluate if HbA_{1c} could be analyzed on stored blood samples in a medical biobank, if HbA_{1c} seemed to be affected by the freezing procedure or time in −80°C storage, and if signs of hemolysis were correlated with HbA_{1c} levels in the general nondiabetic population.

MATERIALS AND METHODS

Study Population

All subjects included in the study participated in the Västerbotten Intervention Program (VIP). VIP is a health program with the aim of reducing the incidence of cardiovascular disease (CVD) and diabetes in the county of Västerbotten. All inhabitants are invited to VIP at the age of 40, 50, and 60 years without any exclusion criteria. In VIP, a standardized OGTT is performed together with analysis of other biomedical risk factors for CVD and diabetes. Different cohorts of VIP have been used in the study, and the subjects in each analysis are defined below. All subjects in our study have performed an OGTT after overnight fasting and were found to be free from diabetes. The glucose analyses were performed on Reflotron benchtop analyzers (Boehringer Mannheim, Mannheim, Germany).[11]

Analysis and Test of Stability of HbA_{1c}

Whole EDTA-anticoagulated blood was analyzed for HbA_{1c} content by cation exchange high-performance liquid chromatography (HPLC) in a TOSOH A1c 2.2 glycohemoglobin analyzer HLC-723Ghb apparatus (TOSOH, Tokyo, Japan), using the software of the manufacturer. The apparatus was calibrated against the Mono-S HPLC procedure, which is a designated comparison method. The reference interval of healthy persons is given as 3.6% to 5.0% below 50 years and 3.9% to 5.3% over 50 years and the percent of coefficient variation (CV%) for the method was <3%. Fresh samples were diluted automatically in the apparatus, whereas frozen samples were diluted manually with "Heamolysis stabilizing solution" containing EDTA and cyanide (TOSOH). Results are presented with 1 decimal.

We estimated the stability of HbA_{1c} during different freezing conditions in the following 3 ways. The basic characteristics are presented in Table 1. All frozen samples were kept at −80°C. First, to study if freezing affected HbA_{1c}, fresh samples (group 1a) were collected consecutively in VIP. A total of 114 random samples from group 1a was first analyzed when they were fresh (group 1b). These samples were thereafter frozen and thawed and reanalyzed for HA_{1c} after 1 to 4 months (group 1c) to evaluate the effect of short-term freezing. Second, to evaluate long-term stability, we compared the HbA_{1c} results for fresh blood samples (group 1a) with the HbA_{1c} results for long-term stored VIP samples (group 2) from randomly selected nondiabetic participants in VIP.[12] Third, we analyzed HbA_{1c} on frozen samples

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Table 1. Basic Characteristics and Comparison of HbA_{1c} (%) Analyzed on Fresh Samples (group 1a), Subgroup of Group 1a (group 1b) Reanalyzed After Short Time Storage (group 1c), Long Time Stored Samples (group 2), and Samples Collected and Stored in a Continuous Manner (group 3) (n, means ± SD)

	Subgroups				
Variable	1a	1b	1c	2	3
n (male/female)	174 (86/88)	114 (54/60)	114	120 (59/61)	483 (283/200)
Age (yr)	48.5 ± 8.0	48.2 ± 8.0	48.2 ± 8.0	47.3 ± 7.8	52.6 ± 6.6
Storage time	-	-	2 mo ± 1.2	12.5 years ± 1.0	8.4 years ± 2.5
HbA_{1c} (%)	4.25 ± 0.39	4.21 ± 0.39	4.21 ± 0.41	4.19 ± 0.43	4.46 ± 0.41

from randomly collected participants in VIP free from diabetes whose samples had been stored in a continuum of 2 to 12 years (group 3) to evaluate if differences in storage time affected the HbA_{1c} values.

Effect of Hemolysis on HbA_{1c}

To evaluate effects of variation in erythrocyte survival length on HbA_{1c} levels, variables for analysis of hemolysis were determined on 75 (31 men and 44 women; mean age, 47.1 years; SD 7.6) consecutively sampled subjects in VIP. B-reticulocytes and total S-bilirubin were determined on a Sysmex XE-2100 apparatus (Sysmex, Kobe, Japan) and a Vitros 950 apparatus (Ortho-Clinical Diagnostics, Rochester, NY), respectively. S-haptoglobin was analyzed by a immunoturbidimeric method on a Hitachi 911 apparatus (Roche Diagnostics, Basel, Switzerland).

Statistics

Results are presented as n, mean values (95% confidence interval (CI)), SD, and *P* values. Tests used were the Kolmogorov-Smirnov test, Students *t*-test, analysis of variance (ANOVA) with Bonferroni post hoc test, paired samples *t* test, and Pearson's correlation test. A *P* value of less than .05 was considered to be significant and the null-hypothesis is rejected unless stated otherwise.

RESULTS

Basic Characteristics and Effect of Sex and Age on HbA_{1c}

The distribution of HbA_{1c} in groups 1a, 1c, and 2 is shown in Fig 1A through C. One subject with a very low HbA_{1c}, 1.8%, was omitted. HbA_{1c} was normally distributed in fresh samples, short-term, and long-term-stored samples, but not in the continuous group. Based on the figures in Table 2, the ANOVA test showed that there was a significant trend of higher HbA_{1c} values with increasing age in both men and women (fresh samples $P = .002$ and $P = .007$, long-term stored samples $P = .04$ and $P < .001$, continuously collected samples $P = .001$ and $P = .03$, men and women, respectively). Analyzing differences in HbA_{1c} between the sexes showed no differences except in fresh samples collected in 50-year-old subjects, in which men had higher HbA_{1c} levels than women ($P = .039$).

Comparison of HbA_{1c} Analyzed on Fresh, Short-Term, Long-Term Frozen Blood Samples, and Samples Collected in a Continuous Manner

Comparison of HbA_{1c} values between 114 fresh (group 1b) and short time frozen samples (group 1c) revealed a high correlation ($r^2 = 0.99$) between the 2 measurements as seen in Fig 2, and there was no significant difference in HbA_{1c} mean values as seen in Table 1. Comparing fresh samples with long time stored samples did not reveal any significant differences. Subjects in the continuous group (group 3) were older than subjects in the other 3 groups, which explained the higher HbA_{1c} values. Within the continuous group there was no trend of time in −80°C affecting HbA_{1c} (ANOVA $P = .58$).

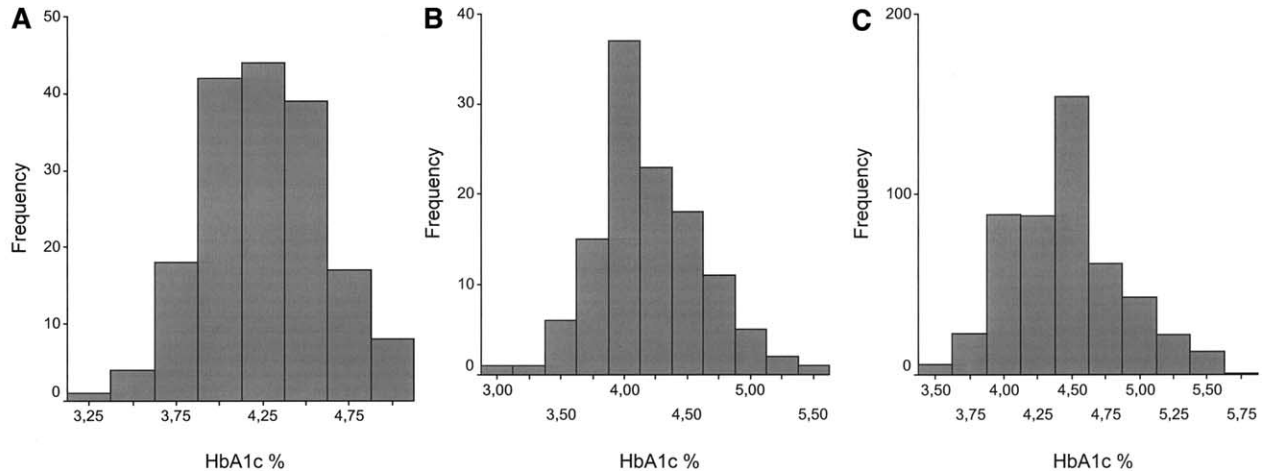

Fig 1. (A) Distribution of HbA_{1c} in fresh samples (group 1a), (B) long-term stored samples (group 2), and (C) samples stored in a continuous manner (group 3).

Table 2. Distribution of HbA_{1c} (%) Analyzed on Fresh Samples (group 1a), Long Time Stored Samples (group 2), and Samples Collected in a Continuous Manner (group 3) in the Different Age Groups Divided by Sex

	Fresh (1a)		Long-Term Stored (2)		Continuous (3)	
	M (n = 86)	F (n = 88)	M (n = 59)	F (n = 61)	M (n = 283)	F (n = 200)
40 yr	4.16 (4.04–4.28)	4.07 (3.98–4.16)	4.12 (4.01–4.24)	3.93 (3.75–4.11)	4.29 (4.17–4.41)	4.39 (4.08–4.71)
50 yr	4.41 (4.29–4.54)	4.16 (3.95–4.37)	4.22 (4.03–4.40)	4.26 (4.03–4.49)	4.43 (4.36–4.49)	4.42 (4.34–4.50)
60 yr	4.43 (4.31–4.56)	4.41 (4.28–4.55)	4.44 (4.19–4.70)	4.53 (4.37–4.69)	4.57 (4.47–4.66)	4.57 (4.49–4.64)

NOTE. Data are means (95% CI).

HbA_{1c} in Relationship to Signs of Hemolysis

As shown in Table 3, we could not observe any significant correlation between HbA_{1c} and parameters indicating hemolysis. Also, when analyzing subjects in the lowest quartile of HbA_{1c} ($HbA_{1c} < 4.0\%$), a group in which hemolysis might be present, we did not find any correlation.

DISCUSSION

In the present study, we are able to present 2 important results. First, we have shown that HbA_{1c} can be analyzed on erythrocytes that have been kept frozen at −80°C up to 14 years. The analysis was made using a standard method, cation exchange HPLC. Our method for comparing frozen erythrocytes from historical samples with fresh erythrocytes may be questioned, because it was not performed on the same subjects. However, this was the only feasible procedure. The optimal protocol would have consisted of drawing samples from subjects, analyzing HbA_{1c} on 1 part of the drawn blood sample, and keeping the other part of the sample frozen for 14 years before analyzing HbA_{1c} again. During these 14 years the analyzing methods have changed several times, which would have made a comparison impossible. We also conclude that the freezing procedure in itself does not affect HbA_{1c}, because we did not find any significant differences in HbA_{1c} after a short freezing period. Also, time at −80°C did not influence the HbA_{1c}, which strengthens our belief that HbA_{1c} can be analyzed on frozen erythrocytes with standard methods. Given this new knowledge, it should be possible to use erythrocytes in medical blood banks, eg, the VIP where blood samples from more than 83,000 subjects are kept frozen. This study has given us means to further test if elevated HbA_{1c} in a banked specimen could be of value in epidemiologic studies of diabetes,[13] cardiovascular diseases,[14] and cancer.[15]

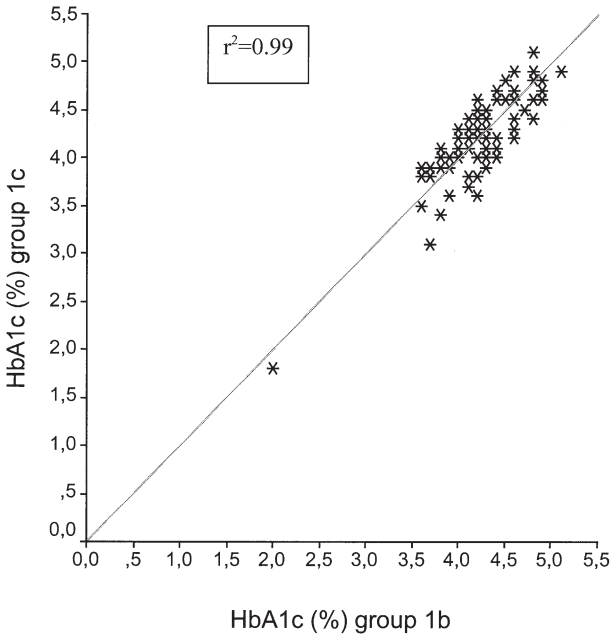

Fig 2. Correlation between HbA_{1c} analyzed on 114 fresh samples (group 1b) and HbA_{1c} on the same samples (group 1c) after being stored at −80°C for a mean time of 2 months and then reanalyzed. Note that the major part of the data pairs close to the correlation line is hidden due to crowding.

It should be emphasized that our conclusions are made after storing samples at a very low temperature, −80°C. Also, our method is based on the TOSOH software and their stabilizing solution. Analyzing HbA_{1c} on frozen samples maybe also could be performed with the Mono-S method as has been done by Borg et al.[16] In an effort to standardize the HbA_{1c} methods in the United States, the National Glycohemoglobin Standardization Program (NGSP) has been relying on sample stability at −80°C degrees for controls, which corroborates our finding.[17]

Because glycation is a continuos process, HbA_{1c} values are influenced by the survival length of erythrocytes. To study the effect of differences in erythrocyte survival lengths on HbA_{1c}, we analyzed variables used for detection of hemolytic disease; B-reticulocytes, S-bilirubin, and S-haptoglobin. No significantly altered values were detected, and there was no correlation with HbA_{1c} values. With the rather crude methods used in the study, no effects of variations in erythrocyte survival length on the HbA_{1c} variability could be detected and we, therefore, suggest that hemolytic disease influencing HbA_{1c} values was

Table 3. Correlation Between HbA_{1c}, Blood Reticulocytes, Serum Haptoglobin, and Serum Bilirubin Analyzed on 75 Fresh Blood Samples (Pearson's correlation coefficient)

	HbA_{1c}	B-Reticulocytes	S-Haptoglobin	S-Bilirubin
HbA_{1c}	1	0.09	−0.03	−0.037
P value		NS	NS	NS
Reticulocytes	0.09	1	−0.10	0.20
P value	NS		NS	NS
Haptoglobin	−0.03	−0.10	1	−0.12
P value	NS	NS		NS
Bilirubin	−0.04	0.20	−0.12	1
P value	NS	NS	NS	

Abbreviation: NS, not significant.

uncommon in the study population. It has been reported previously that low HbA_{1c} could be a result of hemolysis.[9,10] These studies are all small case reports or studies made in subjects with known hemolytic disorders. Our study consisted of subjects from the general population where hemolytic disorders are rare, and we could not find any significant affect of hemolysis parameters on HbA_{1c}. Hemoglobinopathies have been reported to raise methodologic problems when determining HbA_{1c}.[18,19] The abnormal hemoglobin alters the normal glycosylation and often induces a low level of hemolysis. So, in the individual case where an unexpected low HbA_{1c} value is found, a hematologic investigation would be of interest.

In conclusion, in our study, we have shown that HbA_{1c} can be analyzed on erythrocytes frozen at −80°C, and that the time in deep freeze does not significantly affect HbA_{1c}. Therefore, we suggest that studies can be performed on historical blood samples to study HbA_{1c} as a potential predictive tool of future diabetes.

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