Trimester-specific reference intervals for haemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}) in pregnancy

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Abstract

Background: Diabetes in pregnancy imposes additional risks to both mother and infant. These increased risks are considered to be primarily related to glycaemic control which is monitored by means of glycated haemoglobin (HbA\textsubscript{1c}). The correlation of HbA\textsubscript{1c} with clinical outcomes emphasises the need to measure HbA\textsubscript{1c} accurately, precisely and for correct interpretation, comparison to appropriately defined reference intervals. Since July 2010, the HbA\textsubscript{1c} assay in Irish laboratories is fully metrologically traceable to the IFCC standard. The objective was to establish trimester-specific reference intervals in pregnancy for IFCC standardised HbA\textsubscript{1c} in non-diabetic Caucasian women.

Methods: The authors recruited 311 non-diabetic Caucasian pregnant (n=246) and non-pregnant women (n=65). A selective screening based on risk factors for gestational diabetes was employed. All subjects had a random plasma glucose <7.7 mmol/L and normal haemoglobin level. Pregnancy trimester was defined as trimester 1 (T1, n=40) up to 12 weeks +6 days, trimester 2 (T2, n=106) 13–27 weeks +6 days, trimester 3 (T3, n=100) >28 weeks to term.

Results: The normal HbA\textsubscript{1c} reference interval for Caucasian non-pregnant women was 29–37 mmol/mol (Diabetes Control and Complications Trial; DCCT: 4.8%–5.5%), T1: 24–36 mmol/mol (DCCT: 4.3%–5.4%), T2: 25–35 mmol/mol (DCCT: 4.4%–5.4%) and T3: 28–39 mmol/mol (DCCT: 4.7%–5.7%). HbA\textsubscript{1c} was significantly decreased in trimesters 1 and 2 compared to non-pregnant women.

Conclusions: HbA\textsubscript{1c} trimester-specific reference intervals are required to better inform the management of pregnancies complicated by diabetes.

Keywords: glycated haemoglobin (HbA\textsubscript{1c}); pregnancy; reference intervals; trimester.

Introduction

Mothers and infants of pregnancies complicated by diabetes are at increased risk of antenatal delivery and post-natal complications (1–4). Mothers with pre-gestational diabetes type 1 or type 2 entering pregnancy have an increased chance of developing pregnancy-induced hypertension and/or pre-eclampsia. They are also at increased risk of developing polyhydramnios and being delivered by caesarean section. Infants of mothers with pre-gestational diabetes are at increased risk of a congenital malformation or being born large for gestational age or small for gestational age or succumbing to a stillbirth or perinatal death. The risk of admission to a special care baby unit is 4-times greater than the background population and these infants are at risk of neonatal complications, such as hypoglycaemia, respiratory distress syndrome and jaundice (3). These poor outcomes in diabetic patients are considered to be primarily related to glycaemic control and this is monitored longitudinally through pregnancy by means of glycated haemoglobin (HbA\textsubscript{1c}).

The correlation between HbA\textsubscript{1c} levels with clinical outcomes emphasises the need to measure HbA\textsubscript{1c} accurately and precisely. In laboratory medicine, reference intervals are the most common decision support tool used for interpretation of numerical results. As the interpretation of laboratory results and consequent patient management may be guided by comparisons to these intervals, the quality of the reference interval can be as important as the result itself (5). HbA\textsubscript{1c} levels are known to change throughout normal pregnancy (6–9). During the first trimester of pregnancy there is a decrease in the mean fasting and post-prandial blood glucose values (10, 11). Consequently, erythrocytes are exposed to a reduced glucose milieu resulting in less glycation of haemoglobin (12). In addition, it has been demonstrated that the turnover of red blood cells is increased in normal pregnancy, a factor which also contributes to lower HbA\textsubscript{1c} values (13). Together, these findings suggest that to ensure optimum patient management in pregnancy complicated by diabetes, trimester-specific reference intervals for HbA\textsubscript{1c} should be employed.

In 2002 the International Federation of Clinical Chemistry (IFCC) published a reference method for the measurement of HbA\textsubscript{1c} which is now the means for the uniform standardisation of HbA\textsubscript{1c} assays worldwide (14). In Ireland the Health Service Executive (HSE) is leading the national implementation of the International Standardisation of HbA\textsubscript{1c} measurement. The primary goal is that the HbA\textsubscript{1c} assay in all Irish laboratories will
to be fully metrologically traceable to the IFCC standard, permitting \( \text{HbA}_1c \) to be reported in IFCC units (mmol/mol) and derived Diabetes Control and Complications Trial (DCCT)/National Glycohemoglobin Standardisation Program (NGSP) units (%) using the IFCC-DCCT/NGSP master equation (15). The master equation, NGSP=0.09418*IFCC(mmol/mol) + 2.152 links IFCC results to clinically meaningful \( \text{HbA}_1c \) results from the DCCT and the United Kingdom Prospective Diabetes Study (UKPDS). It also provides the NGSP with traceability to a higher order reference measurement method (16). This best practice initiative will mean that \( \text{HbA}_1c \) is measured specifically and reproducibly and will enable the use of international reference intervals and harmonisation of medical decision or “target” values.

The aim of this study is to establish trimester-specific reference intervals in pregnancy for IFCC standardised \( \text{HbA}_1c \) in non-diabetic Caucasian women which would be useful in discussions on the ideal \( \text{HbA}_1c \) in pregnancy.

**Materials and methods**

For this study, healthy non-diabetic pregnant and non-pregnant women were randomly selected through our Outpatient Phlebotomy Department and Antenatal Clinics (including midwife led clinics) as well as a number of General Practices situated in County Galway. A selective screening policy based on risk factors for gestational diabetes was employed (17). Pregnancy trimester was defined as T1 [up to 12 weeks +6 days (n=40)], T2 [13–27 weeks +6 days (n=106)] and T3 [≥28 weeks to term (n=100)].

Following informed consent and at the time of routine blood testing, non-fasting whole blood (10 mL) was drawn into potassium ethylenediamine tetra-acetic acid for the measurement of haemoglobin and glycated \( \text{Hb} \) and into fluoride oxalate specimen tubes for (random) plasma glucose analysis.

Subjects were included in this study based on clinical assessment of wellbeing, random plasma glucose (RPG) ≤7.7 mmol/L, and glycated \( \text{Hb} \) in non-pregnant controls, 118–150 g/L and in the pregnant cohort, T1: 116–139 g/L, T2: 97–148 g/L and T3: 95–150 g/L (18). The baseline demographics of the study cohorts are detailed in Table 1.

Ethical Approval for this study was obtained from Shaun T. O’Keeffe of the Clinical Research Ethics Committee, Unit 4, Merlin Park Hospital, Galway, Ireland on 12/4/2010.

**Analytical methods**

\( \text{HbA}_1c \) \( \text{HbA}_1c \) was assayed using the Menarini HA 8160 automated haemoglobin analyser. The method principle is based on reverse phase cation exchange chromatography and is fully traceable to the IFCC standard in accord with the European Union directive 98/79/EC on in vitro diagnostic (IVD) medical devices. Traceability is achieved using calibrators provided by Menarini. The \( \text{HbA}_1c \) values of these calibrators are assigned using two IFCC Secondary Reference Methods in an approved network laboratory.

The between-run (n=20) analytical coefficient of variation (CV%, %) at a mean \( \text{HbA}_1c \) of 42 mmol/mol (derived DCCT 6%) and 100 mmol/mol (derived DCCT 11.4%) was 2.0% and 1.3%, respectively. The mean bias of the assay as determined by proficiency testing through the Irish External Quality Assessment Scheme (IEQAS) was −2.36% (DCCT units: –2.1%) when compared to values assigned by the IFCC Reference Laboratory. The target \( \text{HbA}_1c \) concentrations on which the assay bias was assessed were 31 mmol/mol (derived DCCT 5.0%), 76 mmol/mol (derived DCCT 9.1%) and 62 mmol/mol (derived DCCT 7.9%), respectively. The current IEQAS \( \text{HbA}_1c \) (laboratory analysis) acceptance limits for bias are ±5% per specimen/±8% per distribution (DCCT units are ±4% per specimen/±6% per distribution) (19).

**Glucose** Plasma glucose was measured using the Roche Modular Chemistry Systems. The method principle is enzymatic, utilising hexokinase which catalyzes the phosphorylation of glucose to glucose-6-phosphate (20). The between-run (n=20) CV% at a mean plasma glucose of 2.97 mmol/L and 18.88 mmol/L was 1.9% and 1.5%, respectively. Assay bias was determined by proficiency testing through the United Kingdom National External Quality Assessment Scheme (UKNEQAS). The target plasma glucose concentrations ranged from 2.68 to 16.91 mmol/L. The mean bias of the assay was +0.8% (acceptance limit ±5%).

**Haemoglobin** \( \text{Hb} \) was measured using the Siemens Advia 120 Haematology system. Flow cytometry and light scattering is used to derive red blood cell (RBC) volume and cell haemoglobin concentration on a cell-by-cell basis (21). The between-run (n=20) CV% at a mean haemoglobin concentration of 58 g/L, 128 g/L and 166 g/L was <1.1%. Analytical bias was appraised at \( \text{Hb} \) concentrations which ranged from 80 to 157 g/L. The mean bias of the assay as determined by the UKNEQAS proficiency testing scheme was 0.47% (acceptance limit <1.0%).

**Statistical analysis**

The Minitab Release version 15 statistical software package was used to illustrate the data. The frequency distributions for \( \text{HbA}_1c \) in non-diabetic Caucasian pregnant and non-pregnant women were established. Outliers were assessed in accordance with the criteria proposed by Dixon (22) and Reed et al. (23). This approach to statistically significant outliers is supported by the IFCC working group (24). To determine the reference intervals, the IFCC non-parametric method, which does not assume a Gaussian type distribution, was employed. For each analyte assessed, the lower and upper reference

<table>
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<th>Table 1</th>
<th>Baseline demographics of non-diabetic Caucasian pregnant and non-pregnant women.</th>
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<tr>
<td></td>
<td>Non-pregnant (n=65)</td>
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<tr>
<td>Age, years</td>
<td>Mean (range)</td>
</tr>
<tr>
<td>28.4 (18–49)</td>
<td>32.3 (24–45)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.7 (18.6–33.4)</td>
</tr>
<tr>
<td>RPG, mmol/L</td>
<td>4.7 (3.2–7.1)</td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>132 (118–150)</td>
</tr>
</tbody>
</table>
limits were estimated at the 2.5th and the 97.5th percentiles, respectively (24). The Kruskal-Wallis test was used to compare the medians between the groups (p < 0.05 is considered significant). The Dunn’s post-test was applied to correct for multiple comparisons.

Results

The frequency distribution of HbA₁c results was non-Gaussian in all cohorts (exception of T1 subjects) according to the Anderson-Darling normality test, p < 0.05. The reference interval was defined by the IFCC as the interval between and including the upper and lower reference limits. This was estimated to enclose 95% of the values for the population from which the reference individuals were chosen. The non-parametric method does not assume a Gaussian-type distribution and was used to establish the reference intervals in this study.

Using the statistical approach described above, we established the normal reference interval for HbA₁c in healthy Caucasian non-pregnant women (n = 65) to be 29–37 mmol/mol (DCCT: 4.8%–5.5%). In normal pregnancy, we determined the HbA₁c reference intervals to be as follows; trimester 1 (n = 40) to be 24–36 mmol/mol (DCCT: 4.3%–5.4%); trimester 2 (n = 106) to be 25–35 mmol/mol (DCCT: 4.4%–5.4%) and trimester 3 (n = 100) to be 28–39 mmol/mol (DCCT: 4.7%–5.7%), Table 2.

Although the median HbA₁c values for all groups demonstrated modest variation, the difference in HbA₁c between the groups was highly significant, p < 0.0001. The reference interval for HbA₁c in late pregnancy (T3) differed little from that of healthy non-pregnant women and comparison of the median HbA₁c of these two groups did not reach statistical significance, p > 0.05. In trimesters 1 and 2 of normal pregnancy the reference intervals were almost equivalent, p-value > 0.05. The latter reference intervals are lower and shifted to the left (Figure 1) when compared to both those of non-pregnant women and women in late pregnancy (T3), statistical significance is reached with p < 0.01 and p < 0.001, respectively (Table 3).

Discussion

Glycaemic control as assessed through the measurement of HbA₁c is central to managing the care of people with diabetes. The clinical usefulness of this test was not fully appreciated

<table>
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<th>Table 2</th>
<th>Reference intervals for HbA₁c in non-diabetic Caucasian pregnant and non-pregnant women.</th>
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<tbody>
<tr>
<td>Variable</td>
<td>Non-pregnant (n=65) (median)</td>
</tr>
<tr>
<td>HbA₁c, derived DCCT, %</td>
<td>4.8–5.5 (5.2)</td>
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</tbody>
</table>

| Figure 1 | HbA₁c in non-diabetic Caucasian pregnant and non-pregnant women. |

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Dunn’s post-test in non-diabetic Caucasian pregnant and non-pregnant women.</th>
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<tr>
<td>Comparison of median HbA₁c values</td>
<td>Significant level p &lt; 0.05</td>
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<tr>
<td>Non-pregnant vs. Trimester 1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Non-pregnant vs. Trimester 2</td>
<td>&lt;0.001</td>
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<tr>
<td>Non-pregnant vs. Trimester 3</td>
<td>&gt;0.05</td>
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<tr>
<td>Trimester 1 vs. Trimester 3</td>
<td>&lt;0.01</td>
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<tr>
<td>Trimester 1 vs. Trimester 2</td>
<td>&gt;0.05</td>
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<td>Trimester 2 vs. Trimester 3</td>
<td>&lt;0.001</td>
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until the publication of two seminal trials, the USA Diabetes Control and Complications Trial for people with type 1 diabetes in 1993 and the UK Prospective Diabetes Study for people with type 2 diabetes in 1998 (25, 26).

Following the introduction of HbA1c assays into routine use it became apparent that there was a significant difference in results produced by different laboratories (27). On publication of the DCCT study, the issue of international standardisation of glycated haemoglobin measurements became an important objective for scientists and clinicians alike (25). The lack of international standardisation resulted in several countries developing national standardisation programs, e.g., National Glycohemoglobin Standardisation Program (NGSP) in the USA.

A common feature of these national programs is the absence of internationally recognised and accepted reference materials and measurement procedures to assure the accuracy and comparability of HbA1c measurements globally (28). To address this, the IFCC established a Working Group on HbA1c standardisation with the aim to develop a complete reference measurement system based on the concepts of metrological traceability. In 2002 the IFCC published a reference method for the measurement of HbA1c, which is now the method for the uniform standardisation of HbA1c assays worldwide (14). The effect of which is to link the measurement result from a patient sample to a commonly accepted reference when using different types of measurement equipment in different locations over time. The import of this initiative is far-reaching and will permit the use of international reference intervals and harmonisation of medical decision values.

From July 2010 all laboratories in the Republic of Ireland implemented the IFCC standardisation of HbA1c assays. Given the latter initiative, we set out to determine IFCC traceable reference intervals in normal pregnancy with the objective of defining the ideal HbA1c so as to better inform the management of diabetic pregnant women.

We established the reference interval for HbA1c in healthy Caucasian non-pregnant women to be 29–37 mmol/mol, median 33 mmol/mol (DCCT: 4.8%–5.5%). This is almost identical to that established by the IFCC of 29–38 mmol/mol, mean 33 mmol/mol in their study of 120 non-diabetic Danish subjects (aged 35–55 years) with normal glucose tolerance (14).

Our results show that HbA1c in healthy pregnant women without diabetes or anaemia are lower in T1 (up to 12 weeks +6 days) and T2 (13–27 weeks +6 days) than those of non-diabetic non-pregnant women of comparable age (Figure 1). Our findings are in agreement with those of Nielsen et al. (6), Mosca et al. (7), Radder and van Roosmalen (8) and O’Kane et al. (9). However, the HbA1c assays used in these studies were DCCT aligned. To our knowledge this is the first study to establish reference intervals in normal pregnancy with statistical elaboration in accord with IFCC recommendations using an HbA1c assay which is metrologically traceable to the IFCC reference method. The goal for HbA1c in pregnant women with diabetes is usually achieved with reference to that of non-diabetic non-pregnant women. Our study suggests that this is inappropriate and that trimester-specific reference intervals for HbA1c during pregnancy should be used to manage pregnancy complicated by diabetes.

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Conflict of interest statement

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References