HbA1c (glycated haemoglobin) (blood)

1 Name and description of analyte

1.1 Name of analyte :
HbA_{1c}

1.2 Alternative names
Beta N-(1-deoxyfructos-1-yl) haemoglobin (IFCC approved nomenclature); haemoglobin A_{1c}, HbA1c, glycated haemoglobin, glycohaemoglobin, GHb.

1.3 NLMC code
To follow

1.4 Description of analyte
HbA1c is a modified haemoglobin, with a stable adduct of glucose (covalently linked) to the N-terminal valine of the β chain. Normal adult haemoglobin consists predominantly of HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) (97, 2.5 and 0.5% respectively). About 6% of total HbA is termed HbA_1, which in turn is made up of HbA_{1a1}, HbA_{1a2}, HbA_{1b} and HbA_{1c}. These fractions are defined by their electrophoretic and chromatographic properties, which differ slightly from those of the major component HbA_0, despite the amino acid sequences of HbA_1 and HbA_0 being identical. HbA_{1c} is the most abundant of these fractions and in health comprises approximately 5% of the total HbA fraction. Structural and chemical investigations elucidated that glucose, in the open chain format, binds to the N-terminal to form an aldimine (Schiff base) before undergoing an Amadori rearrangement to form a more stable ketoamine. This is a non-enzymatic process that occurs continuously *in vivo.*

1.5 Function of analyte
No known physiological role

2 Sample requirements and precautions

2.1 Medium in which measured
HbA1c is typically measured in anticoagulated whole blood, usually EDTA but other anticoagulants may be used depending on the principle of the analytical method.

2.2 Precautions re sampling, handling etc.

- Samples are stable at 4 °C for one week according to most manufacturers. A recent study has shown that samples may be stable for up to eight weeks at 4 °C depending on the assay principle. For ion-exchange methods, storage at 4 °C is preferable to -20 °C. For long term storage, -70 °C or lower is recommended.
- EDTA tubes should be filled to capacity.
- There is not known to be a diurnal variation in HbA_{1c} concentrations.

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3 Summary of clinical uses and limitations of measurements

3.1 Uses

1. Monitoring glycaemic control in patients with diabetes
2. Diagnosis of type 2 diabetes mellitus

3.2 Limitations

- HbA_{1c} cannot be used for the diagnosis of diabetes in children, for the diagnosis of gestational diabetes or of type 1 diabetes.
- HbA_{1c} should not be used for the diagnosis or monitoring of patients with diabetes with certain haemoglobinopathies or with disorders which affect red cell lifespan (see 4.5).

4 Analytical considerations

4.1 Analytical methods

The main analytical methods used for the measurement of HbA_{1c} are: cation exchange chromatography, affinity chromatography, immunoassay and capillary electrophoresis. These methods make use of the difference in charge between HbA_{1c} and HbA_0 or the structural differences between glycated and non-glycated forms of haemoglobin.

The European Reference Laboratory External Quality Programme shows that in Europe, some 75% of laboratories use ion-exchange HPLC, 23% immunochemistry, and only a few use affinity chromatography, although this may change with the introduction of new affinity chromatography and capillary electrophoresis analysers to the market in the coming years.

1. Cation exchange chromatography.

Haemoglobins A_{1c} and A_0 have a subtle difference in their isoelectric points and can be separated on this basis. In 1971, Trivelli and co-workers described a separation on shortened ion-exchange columns, in which the fast fractions were removed with 0.055 mmol/L phosphate buffer, pH 6.70 (HbA_{1a} and HbA_{1b} together and HbA_{1c} separately); the remaining haemoglobins (HbA_0 and HbA_2) were removed with 0.15 mmol/L phosphate buffer, pH 6.42. The absorbance of the fractions eluted from the column was measured at 415nm and the fractions expressed as a percentage of the total. Later, automated high-performance liquid chromatography (HPLC) systems were developed. After many generations, several systems (major suppliers: Tosoh, Bio-Rad, and ARKRAY/Menarini) have reached a high level of performance.
These methods do not suffer from interference by the Schiff base or carbamylated haemoglobin but may be prone to interference from haemoglobin variants, which may co-elute with the peaks of interest.

2. Affinity chromatography

Affinity separation of glycated haemoglobin typically utilises *m*-aminophenyl boronic acid and depends on a specific interaction between the glucose on glycated haemoglobin and the immobilised boronic acid. Haemolysate is applied to the affinity column and the GHb that contains coplanar *cis*-diol groups interacts strongly with boronic acid immobilised on an agarose gel. Ionic and hydrophobic forces also contribute to this interaction. The non-glycated haemoglobin elutes directly off the column with the first buffer. After elution of the non-glycated fraction, bound

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haemoglobin can be dissociated by the use of a counter-ligand, which effectively competes with bound glycated haemoglobin for the boronic acid sites on the gel surf ace. The absorbance of the haemoglobin fractions can be measured at 414 nm and the ratio determined.

3. Immunoassay
The antibody is targeted against the β N-terminal glycated tetrapeptide or hexapeptide group. Assay design is variable, ranging from immunoturbidimetry to latex-enhanced competitive immunoturbidimetry and enzymatic detection. There are a number of commercial assays that are applicable to a broad variety of general chemistry analyzers (including those manufactured by Roche, Siemens and Vitros). Immunochemical assays are not affected by problems related to electrical charge and can be adapted easily to use in the routine medical laboratory. However, they all suffer with the general drawback of immunochemistry, i.e. non-linear calibration, which requires multilevel calibration. As stability of the reagent is limited (variably from test to test), relatively frequent recalibration is needed. Also, to quantitate HbA_{1c}, as a ratio, total haemoglobin is measured separately, using a different analytical principle that introduces additional uncertainty to the outcome.

4. Capillary electrophoresis
Capillary electrophoresis uses the principle of liquid-flow capillary electrophoresis in free solution. With this technique, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electroosmotic flow. The separation of the different haemoglobin fractions takes place in silica capillary tubes of internal diameter <25μm, and the migration is performed at the high voltage (e.g. 9800 volts) under tight temperature control using a Peltier device. The haemoglobins are directly detected at a specific absorption wavelength of 414 nm at the cathodic end of the capillary using an optical detector made of a deuterium lamp and optical fibres.

4.2 Reference method
An International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) working group has developed a reference measurement procedure based on the enzymatic cleavage of the intact haemoglobin molecule with endoproteinase Glu-C to obtain the β-N-terminal hexapeptides of HbA_{1c} and HbA_0. This avoids the heterogeneity created by introducing modifications of other glycation sites on the haemoglobin molecule. The peptides can then be separated by reverse-phase HPLC, and quantified by electro-spray ionization-mass spectrometry (ESI-MS) or by capillary electrophoresis (CE), as illustrated in figure below.

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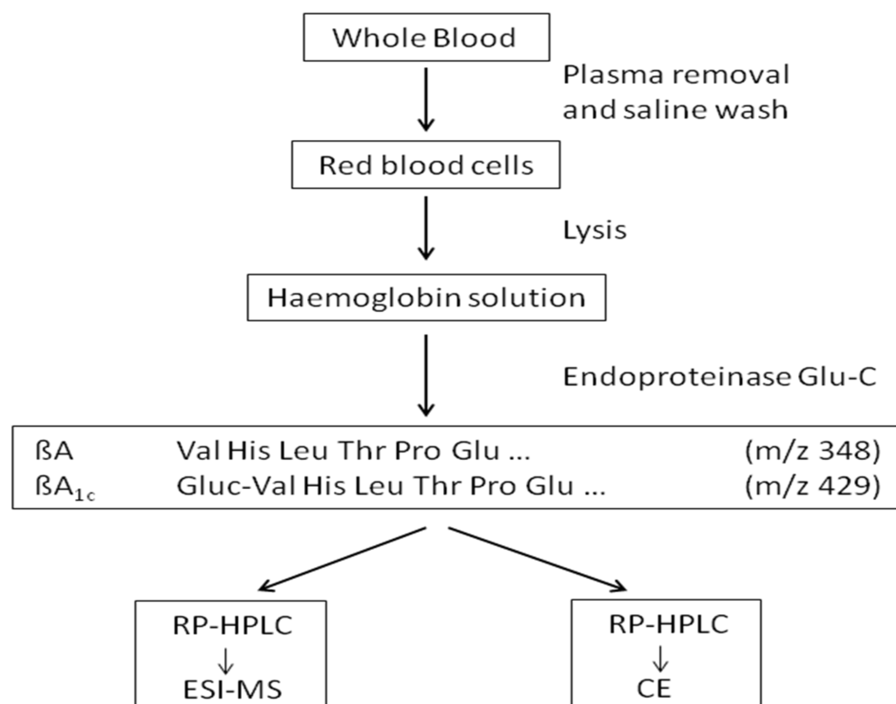

4.3 Reference materials

The IFCC working group on HbA_{1c} standardisation has produced the following primary reference materials, which are now banked at the Institute for Reference Materials and Measurements (IRMM):

- nonglycated haemoglobin A (>99.5 % pure) reference number IRMM/IFCC 467
- β-N-terminal glycated haemoglobin A (>98.5 % pure) reference number IRMM/IFCC 466.

4.4 Interfering substances

Interference is method-dependent.

1. Variant haemoglobins Influence on HbA_{1c} analysis has been reported from HbF HbS, HbC, HbE, HbD and by other variant haemoglobins. Variant haemoglobins with differing chromatographic mobility may co-elute with the peaks of interest and interfere with accurate measurement using ion exchange chromatography. It is not known to what extent the alteration in structure of variant haemoglobins affects the relative glycation rate of HbA_{1c} as a confounding factor. The National Glycohemoglobin Standardization Program maintains a list of analysers with known interferences from haemoglobin variants and can be found at http://www.ngsp.org/interf.asp

2. Jaundice and hyperlipidaemia

Severely icteric specimens may give falsely elevated HbA_1 values with methods relying on charge separation if whole blood haemolysates are used, since bilirubin migrates with the fast haemoglobin and absorbs at the detecting wavelength.

Hyperlipidaemia can also cause false elevation of HbA_1, since lipids elute in the first HbA_1 fraction and absorb at 415 nm; this issue is method specific. It could be magnified if analysis were performed on post-prandial samples. Since hyperlipidaemia is a relatively common finding in diabetic patients, this limitation is important.

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3. Acetylation by aspirin
Aspirin modifies several sites, probably lysine residues, on both the α and β chains of HbA. Acetylation of lysine residues with aspirin confers a negative charge on the modified protein. The modified haemoglobin has altered electrophoretic and chromatographic (ion exchange) properties, migrating ahead of HbA_0, like HbA_1. Although there is *in vitro* evidence to show interference by acetylation it is unclear to what degree this is an issue *in vivo*.

4. Carbamylation by urea in renal failure
Elevated quantities of both HbA_1 and HbA_{1c}-like haemoglobins have been reported in patients with uraemia; this effect is method-specific. Newer methods are designed to obviate this interference. It is also worth noting that patients with renal failure are often predisposed to anaemia and have reduced red cell survival, which will also have an effect on the quantity of HbA_{1c}. These patients are frequently treated erythropoietin or a related substance, which also affects red cell half life. Further information is available at http://www.ngsp.org/interf.asp

4.5 Sources of error
Disorders causing shortened red cell survival (e.g. haemolytic anaemias) will result in decreased values for glycated haemoglobin. In contrast, higher values can occur in people with a longer red cell life-span, e.g. in vitamin B12 or folate deficiency. The percentage of glycated haemoglobin is also increased by the increased red cell survival after splenectomy. Iron deficiency anaemia may also result in extended red cell life-span and an increase in HbA_{1c}. Blood transfusion will bias the percentage of glycated haemoglobin towards that of the transfused blood; this is particularly relevant as transfusion bags contain a high glucose concentration.

5 Reference intervals and variance

5.1.1 Reference interval (adults)
Individuals displaying normoglycaemia would be expected to have values below 39mmol/mol (5.7%). At values of 39–46mmol/mol, individuals may be at increased risk of developing diabetes. For information on diagnosis see section 6.1(2).

5.1.2 Reference intervals (others)
As above

5.2.1 Extent of variation
Data are limited to small studies but the following information is derived from a study by Braga *et al.*

5.2.1.1 Interindividual CV 7.1%
5.2.1.2 Intraindividual CV 2.5%
5.2.1.3 Index of individuality 0.35
5.2.1.4 CV of method
There are few published data as to the analytical performance required for HbA_{1c} methods used for diagnosis, but as a minimum the within-laboratory imprecision should be <3% CV and between-laboratory agreement must be <5% CV based on SI units (mmol/mol) (for explanation see Weykamp *et al*). Certain methods, in particular point of care devices, may not achieve minimum requirements for diagnosis.

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5.2.1.5 Critical difference 9.5%

5.3 Sources of variation

The results of the Diabetes Prevention Program (3819 individuals ≥25 years old with IGT) indicate that ethnicity is an independent factor in determining HbA1c. 'Adjusting for glucose concentration and a range of other factors, mean HbA1c levels were 5.78% for whites, 5.93% for Hispanics, 6.00% for Asians, 6.12% for American Indians, and 6.18% for blacks ($p < 0.001$).'

The degree of glycaemia is known to alter with age. A meta analysis of data from the Framingham Offspring Study and the National Health and Nutrition Examination Survery showed that in non-diabetic patients there is an approximate increase of 7mmol/mol HbA1c (0.6%) between the ages of 40 and 70 years.

6 Clinical uses of measurement and interpretation of results

6.1 Uses and interpretation

1. Monitoring glycaemic control in patients with diabetes.

NICE guidelines for type 1 diabetes (CG15) July 2004.

- Children and young people and adults with type 1 diabetes should aim to obtain an HbA_{1c} concentration of <58 mmol/mol (<7.5%) without frequent disabling hypoglycaemia and maximising quality of life.
- Children and young people with type 1 diabetes should be offered testing of their HbA_{1c} concentrations 2–4 times per year (more frequent testing may be appropriate if there is concern about poor glycaemic control).
- In adults, monitoring of glycaemic control should be assessed by measurement of HbA_{1c} every 2–6 months, depending on:
  - achieved level of blood glucose control
  - stability of blood glucose control
  - change in insulin dose or regimen.
- Where there is evidence of increased arterial risk (identified by a raised albumin excretion rate, features of the metabolic syndrome, or other arterial risk factors), people with type 1 diabetes should be advised that achieving lower HbA_{1c} values (for example, 48 mmol/mol (6.5%) or lower) may be of benefit to them.

NICE guidelines for type 2 diabetes (CG66, May 2008, partially updated by CG87, May 2009)

When setting a target glycated haemoglobin (HbA_{1c}):

- involve the person in decisions about their individual HbA_{1c} target value, which may be above that of 48 mmol/mol (6.5%) set for people with type 2 diabetes in general
- encourage the person to maintain their individual target unless the resulting side effects (including hypoglycaemia) or their efforts to achieve this impair their quality of life
- avoid pursuing highly intensive management to levels of less than 48mmol/mol (6.5%).

Measure the individual's HbA_{1c} concentration at:

- 2–6-monthly intervals (tailored to individual needs) until the blood glucose concentration is stable on unchanging therapy; use a

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measurement made at an interval of <3 months as a indicator of direction of change, rather than as a new steady state

- 6-monthly intervals once the blood glucose concentration and blood glucose-lowering therapy are stable.

Note that these guidelines are currently under review and will be updated in the near future.

2: Diagnosis of type 2 diabetes mellitus

There are currently differences in practice between different countries. In 2009, an International Expert Committee convened by the American Diabetes Association (ADA) concluded that the cut-off for the diagnosis of diabetes should be an HbA_{1c} of ≥48 mmol/mol (≥6.5%). Individuals with an HbA_{1c} of 39-46 mmol/mol (6.0– 6.4%) should be considered at high risk for progression to diabetes; but 'this range should not be considered an absolute threshold at which preventative measures are initiated.'

This recommendation has been endorsed by the World Health Organisation, which states that 'HbA_{1c} can be used as a diagnostic test for diabetes providing that stringent quality assurance tests are in place and assays are standardised to criteria aligned to the international reference values, and there are no conditions present which preclude its accurate measurement. An HbA_{1c} of 48 mmol/mol (6.5%) is recommended as the cut point for diagnosing diabetes. A value of <48 mmol/mol (<6.5%) does not exclude diabetes diagnosed using glucose tests.'

In the UK, guidelines from professional bodies supported by the Departments of Health have been issued recently (see 9.2 John *et al*, 2011), which state that HbA_{1c} can be used for the diagnosis of type 2 diabetes with a cut off of ≥48 mmol/mol. In patients without symptoms this test should be repeated within 2 weeks. If the value in a second sample is <48 mmol/mol, the individual should be treated as high risk and testing repeated in 6 months or sooner if symptoms develop. In symptomatic adults, a single result of ≥48 mmol/mol will suffice. HbA_{1c} must not be used in the following cases:

- *any* symptomatic child or young person (≤18 years)
- symptoms suggesting type 1 diabetes (at any age)
- short duration diabetes symptoms
- patients at high risk of diabetes who are acutely ill
- patients taking medication that may cause rapid glucose rise such as corticosteroids and anti-psychotics
- acute pancreatic damage/pancreatic surgery.

Guidelines are available ahead of print at http://www.ncbi.nlm.nih.giv/pubmed/22957983

6.2 Confounding factors

See sources of error/variation (see 4.5, 5.1.4). It should also be noted that patients with renal failure are often predisposed to anaemia and have reduced red cell survival, which will also have an effect on the quantity of HbA_{1c}. These patients are frequently treated erythropoietin or a related substance, which also affects red cell half life. Further information is available at http://www.ngsp.org/interf.asp

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7 Causes of abnormal results

7.1 High values

7.1.1 Causes

- diabetes mellitus
- splenectomy
- iron deficiency anaemia
- variant haemoglobins.

7.1.2 Investigation

If a high value is not expected clinically, appropriate investigations may include glucose measurements for diabetes, full blood count, ferritin, vitamin B12 and folate for anaemia and Hb electrophoresis for variant haemoglobin investigations.

7.2 Low values

7.2.1 Causes

- hypoglycaemia
- haemolytic anaemia
- insulinoma.
- splenomegaly
- rheumatoid arthritis

7.2.1 Investigation of low values

If a low value is not expected clinically, investigations may include glucose measurements to indentify hypoglycaemia, full blood count including reticulocyte count, serum lactate dehydrogenase activity and/or serum haptoglobin for haemolytic anaemia and a fasting with glucose and insulin measurements if insulinoma is suspected.

7.3 Notes

None

8 Performance

8.1 Sensitivity, specificity etc. for individual conditions

The New Hoorn study showed, that using a cut point of ≥42 mmol/mol (6.0 %), 50% of patients who had tested positive for diabetes using fasting plasma glucose or 2 h post glucose load values would not be positive by HbA_{1c} value. The study authors suggest that at ≥ 40mmol/mol HbA1c had a sensitivity of 72% and specificity of 91% for the diagnosis of diabetes (van't Riet *et al* 2010). The values are similar to those of the NHANES (National Health and Nutrition Examination Survey) study, which reported that HbA_{1c} would only detect 30% of those diagnosed with diabetes by any criteria.

According to the WHO systematic review on the use of HbA_{1c} in the diagnosis of type 2 diabetes, in the DETECT-2 (Evaluation of Screening and Early Detection Strategies for Type 2 Diabetes and Impaired Glucose Tolerance) collaborative study, the optimal cut-points for detecting diabetes-specific retinopathy in all subjects were plasma glucose concentrations of 6.5 mmol/L (fasting) and 12.4 mmol/L (2 h post glucose administration), and 6.3% for HbA_{1c}. At these cut points the areas under the ROC curves, sensitivities and specificities were 0.87, 82% and 81%, respectively for fasting plasma glucose; 0.89, 83% and 83% for 2 h plasma glucose, and 0.90, 86% and 86%, respectively, for HbA_{1c} (WHO, 2011).

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9 Systematic reviews and guidelines

9.1 Systematic reviews

Al-Ansary L, Farmer A, Hirst J *et al.* Point-of-care testing for HbA_{1c} in the management of diabetes: a systematic review and meta analysis. Clin Chem. 2011;57:568-576.

9.2 Guidelines

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9.3 Recommendations

See 9.2

10 Links

10.1 Related analytes

Glucose

10.2 Related tests

Oral glucose tolerance test

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Further References

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