INSULIN ASSAYS AND REFERENCE VALUES

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SUMMARY - Insulin is produced by β cells in pancreatic islets of Langherans via a complex process of proteolytic conversion. A precursor molecule, proinsulin, is transported to the Golgi apparatus where it is packed into secretory granules. Maturation of the secretory granules is associated with conversion of proinsulin to insulin and C-peptide by enzymatic cleavage. Secretion of insulin into the bloodstream is accompanied by the release of small amounts of proinsulins. Insulin immunoassays consist of radioimmunoassays using polyclonal antisera which cross-react with proinsulins, and two-site assays using monoclonal antibodies. These immunometric assays have led to improvements in specificity and sensitivity as compared to radioimmunoassays. To determine reference values and limits, insulinaemia must be measured in normoglycaemic subjects with a normal body weight. Moreover, as insulinaemia is most often measured during stimulation tests, reference values must also be determined for the most common tests such as the oral glucose tolerance test or the intravenous glucose tolerance test. We report the analytical characteristics of insulin assays and review reference values and their interpretation. Wide-scale use of insulin assays remains a subject of research rather than a diagnostic application. Spontaneous hypoglycaemia, a disorder which can be caused by hyperinsulinism, insulinoma, insulin autoimmune syndrome and non-insulin-mediated factors, is almost the only clinical indication for the measurement of plasma insulin. Diabetes is diagnosed solely on the basis of chronic hyperglycaemia. Thus, measurement of plasma insulin has no clinical value in the diagnosis or management of diabetic patients, with the exception of rare cases including the syndrome of severe insulin resistance and abnormalities in β-cell secretory products. Otherwise, insulin measurement is used in experimental investigations to study the pathophysiology of various disorders, especially diabetes. The reference and range of plasma insulin values are not yet clearly established, and the range of concentrations reported in the literature remains unsatisfactory. There is a need to standardise results and thereby improve comparability among studies.

Key-words: Insulin, proinsulin, C-peptide, radioimmunoassay, immuno-noradiometric assay, oral glucose tolerance test, intravenous glucose tolerance test, diabetes, hypoglycaemia.

RÉSUMÉ - Dosages de l’insuline et valeurs de référence.

L’insuline est synthétisée par les cellules β des îlots de Langherans du pancréas sous la forme d’un précurseur, la proinsuline, qui est ensuite transportée dans l’appareil de Golgi où un système enzymatique complexe génère l’insuline et le peptide-C. Ce processus engendre des composés intermédiaires (split-proinsulins) qui, avec la proinsuline intacte, subsistent en faible proportion au côté de l’insuline et du peptide-C dans les granules de sécrétion. La sécrétion d’insuline dans le compartiment vasculaire s’accompagne donc de la libération de faibles quantités de proinsulines. L’insuline est dosée par des techniques radioimmunologiques (RIA) utilisant des anticorps polyclonaux reconnaissant les proinsulines, ou immunométriques (« sandwich ») à l’aide d’anticorps monoclonaux. Les techniques immunométriques sont plus spécifiques et, en général, plus précises et sensibles que les RIA. Pour établir des valeurs de référence, il est nécessaire de déterminer l’insulinaémie chez des sujets normoglycémiques et non obèses. De plus, comme l’insuline est le plus souvent mesurée au cours de test de stimulation, il est nécessaire d’établir des valeurs de référence pour les principaux test utilisés comme l’hypoglycémie provoquée par voie orale ou intra-veineuse. Nous décrivons ici les caractéristiques analytiques des immunodosages de l’insuline et rapportons les valeurs de référence publiées et leur interprétation. Le dosage de l’insuline a très peu d’indications diagnostiques et est surtout utilisé dans le domaine de la recherche, notamment en diabétologie. L’hypoglycémie, qui peut être causée par une sécrétion inappropriée d’insuline (insulinome, hyperinsulinisme, syndrome autoimmun anti-insuline) est quasiment la seule indication en clinique du dosage de l’insuline. Le diabète est défini par la seule présence d’une hyperglycémie chronique et le dosage de l’insuline n’a pas d’indication dans le diagnostic ou le suivi de cette maladie hormis certaines formes rares comme les syndromes d’insulinorésistance majeure, la présence de formes anormales de l’insuline ou de ses précurseurs. Les valeurs de références de l’insuline ne sont pas encore clairement établies, les intervalles des concentrations rapportées sont très variables d’une étude à l’autre. Une amélioration de la standardisation des dosages reste nécessaire, ce qui permettrait ainsi une meilleure comparaison entre les diverses études.

Mots-clés : Insuline, proinsuline, C-peptide, RIA, IRMA, HGPO, IVGTT, diabète, hypoglycémie.
More than 40 years after the first assay was developed by Yalow and Berson [1], the reference values and reference limits of insulinaemia have still not been firmly established, particularly with regard to dynamic tests. This is due to a variety of reasons, including technical progress in assay methods, leading to different results for a given sample; the small size of population samples, preventing meaningful statistical analysis; failure to state the anthropometric characteristics of the reference populations; and different dynamic testing conditions (variable glucose load, etc.).

INSULIN STRUCTURE AND SECRETION

Insulin, which is produced by β cells of pancreatic islets of Langherans, consists of 51 amino acids (5808 Da) contained within two peptide chains: an A chain with 21 amino acids and a B chain with 30. The chains are connected by two disulphide bridges. A precursor molecule, preproinsulin (11,500 Da), is produced in the endoplasmic reticulum and cleaved by microsomal enzymes to yield proinsulin almost immediately after its synthesis. Proinsulin is transported to the Golgi apparatus, where packaging into clathrin-coated secretory granules takes place. Proinsulin consists of a single chain of 86 amino acids (9600 Da) containing 3 disulphide bridges, and includes the A and B chains of the insulin molecule plus a connecting segment of 35 amino acids. Maturation of the secretory granules is associated with loss of the clathrin coating and conversion of proinsulin to insulin and C-peptide by proteolytic cleavage. Two endoproteases, proconvertase 2 and 3, cleave proinsulin at the AC and BC junctions. Further processing by carboxypeptidase H results in the removal of the two pairs of dibasic amino acids located at the two cleaved junctions to give the des forms of partially processed proinsulins, as shown in Figure 1. C-peptide and insulin are produced when enzymatic cleavage is complete at both junctions. As a small amount of proinsulin produced by the pancreas escapes cleavage partially or totally, normal mature secretory granules contain insulin and C-peptide in equimolar quantities, plus 2 to 6% of intact and split proinsulins [2-4].

Thus, secretion of insulin into the bloodstream, which occurs by exocytosis, is accompanied by the release of an equimolar quantity of C-peptide and small amounts of proinsulins. Over 24 h, the estimated total insulin secretion on a standard high-carbohydrate diet is 60 ± 15 IU [4, 5]. Basal insulin secretion, calculated for the period from 2 h to 7 h, is 1.3 ± 0.4 IU/h [5]. Approximately 50% of insulin is removed in a single pass through the liver, while C-peptide and proinsulins are mainly eliminated by the kidneys [5-8]. Around 6% of C-peptide produced by the pancreas is excreted intact in urine [5]. As proinsulins and C-peptide are not removed by the liver, their half-lives are longer (90 min and 20-30 min respectively, versus 3-5 min for insulin) [4, 5, 7, 9]. This allows proinsulins to accumulate in the blood, where they account for 15-20% of the total amount of insulin+proinsulins in the basal state [10-12]. As proinsulin conversion has a preferred sequential route, with cleavage at the BC junction occurring first, the two major proinsulins in plasma are intact proinsulin and des 31,32 proinsulin, far exceeding other barely detectable 65,66 products [10-12]. Proinsulins have 5-10% of the bioactivity of insulin [7].

Insulin levels vary in a complex fashion in both the fed and fasting states. Rapid pulses of insulin (and C-peptide) with a periodicity of 10-15 min, and ultradian oscillations with periods ranging from 1 to 3 hours, have been identified, accounting for oscillations of insulinaemia [13-19]. Different studies have re-
ported a mean amplitude of the rapid insulin pulses of 1.1 mIU/l [13] or 3 mIU/l (relative amplitude 17 and 30% respectively) in the fasting state [16, 18, 19]. This amplitude is approximately doubled after a glucose load, while the amplitude of ultradian oscillations is multiplied by 3 to 4 [15, 16]. In Type 2 diabetes, impaired glucose tolerance (IGT) and insulinoma, rapid pulses, and ultradian oscillations of insulin secretion are altered [18, 19]. During an oral glucose tolerance test (OGTT), insulin secretion is higher in the morning than in the afternoon or evening [20]. Circannual rhythms have also been described, with stronger and earlier insulin secretion (measured during an OGTT) in autumn than in spring [14, 17].

### INSULIN MEASUREMENT

The four basic methods used to measure insulin and its precursors are bioassays [21-23], high-performance liquid chromatography (HPLC) [23-26], the stable isotope dilution mass spectrometry assay [27] and immunoassays (the only ones which can be used routinely). Insulin bioassays, which measure the biological activity of insulin in various animals, are lengthy, relatively imprecise and insensitive, and require a large number of animals. In recent years, they have been used mainly in establishing international standards and in the commercial standardisation of therapeutic insulins. Chromatographic methods, especially HPLC, can be useful for the analysis of pharmaceutical preparations, validation of immunoassays and analysis of insulin from different species, but require large volumes of plasma and are time-consuming.

#### Insulin immunoassays

The first insulin immunoassays were radioimmunoassays (RIA) with polyclonal antisera which cross-reacted with proinsulin. With the development of monoclonal antibodies in the late 1980s, two-site insulin assays were introduced, i.e. immuno-radiometric (IRMA), -enzymometric (IEMA), and -fluorimetric (IFMA) methods [12, 23, 28-33]. These immunometric assays had better specificity and sensitivity than RIA (Table I). The detection limit of the most sensitive assay is < 1 mIU/l with immunometric techniques and ≈ 2 mIU/l with RIA (Table I). Calibration is performed using an international standard. Several standards have been established, first with animal insulin and more recently with human insulin [21]. The latest standard is IRP 83/500, which contains semisynthetic human insulin with a potency of 26 international units per mg (1 IU = 6 nmol = 34.85 μg).

Insulin values given by most IRMA/IEMA methods are around 20 to 40% lower than corresponding RIA values [12, 28-34]. This difference can only be partly explained by the lower specificity of RIA and is not always found. The interassay coefficient of variation is 6% with the best IRMA/IEMA techniques, and can be as high as 10-15% with other methods, especially RIA [12, 28-33].

All RIA methods but one recognise many of the epitopes common to insulin and proinsulins. However, cross-reactivity varies from 40 to 100% according to the polyclonal antibodies used. Specific monoclonal antibodies do not cross-react with intact proinsulin or des 31,32 proinsulin, which together account for almost all circulating proinsulins. On the other hand, all these monoclonal antibody-based assays but one (an in-house method [33]) recognise des 64,65 proinsulin and cannot therefore be considered strictly specific to insulin (Table I). However, as the fasting plasma concentration of des 64,65 proinsulin is < 1 pmol/l [10-12], and no more than a few pmol/l after glucose stimulation (apart from the exceptional case of hyperproinsulinaemia), these immunoassays are often considered to measure insulin alone.

RIAs recognise most animal insulins and human insulin analogs such as Lispro. Immunoassays using monoclonal antibodies generally recognize pork insulin but not rat insulin. Cross-reactivity with other animal insulins and Lispro varies according to the assay (Table I).

#### Analytical interference

The main sources of interference with insulin determination are haemolysis and different types of circulating antibody (e.g. anti-insulin antibodies).

The insulin-degrading enzyme (IDE: EC 3.4.24.56), specific for insulin, is widely distributed in various tissues, including red blood cells. IDE may not play a key role in insulin metabolism, and fundamental questions on its biological role remain to be answered [35]. Recently, IDE was characterised as a peroxisomal protease [35]. As red blood cells contain high levels of IDE, haemolysed samples should not be analysed. Even a small degree of haemolysis can lead to a marked loss of insulin: slight haemolysis (0.5 g/l) significantly reduced the observed insulin level at 20°C, and massive haemolysis (6 g/l) degraded more than 90% of insulin after one hour at 37°C [36-39]. Although the percentage of insulin recovery was lower in IRMA than in RIA, marked insulin loss was also observed with RIA. The difference between the two methods can be explained by the lack of specificity of polyclonal antibodies (which could cross-react with insulin fragments) and/or the degradation of 125I-insulin used as a tracer in RIA. Insulin degradation can be prevented by adding IDE inhibitors in the sample tube before blood collection. The most effective IDE inhibitors are diamide and p-chloromercuriphenylisulphonic acid; lysosomal enzyme inhibitors such as aprotinin and leupeptin have no effect on IDE activity [38, 39].
<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
<th>D.L.</th>
<th>mlU/l intact hPI</th>
<th>Des(31,32)hPI</th>
<th>Des(64,65)hPI</th>
<th>Rat insulin</th>
<th>Pork insulin*</th>
<th>Lispro</th>
<th>Fasting-mlU/l</th>
<th>Reference interval*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI-Insulin RIA SANOFI-Pasteur</td>
<td>RIA</td>
<td>2.5</td>
<td>80</td>
<td>70</td>
<td>100</td>
<td>90*</td>
<td>100</td>
<td>100</td>
<td>5-1.8.5</td>
<td></td>
</tr>
<tr>
<td>Coat-A-Count Insulin DPC-Behring</td>
<td>RIA</td>
<td>2</td>
<td>55</td>
<td>50</td>
<td>55</td>
<td>ND</td>
<td>100</td>
<td>100</td>
<td>0-30 m ± 2 SD</td>
<td></td>
</tr>
<tr>
<td>Human Insulin specific RIA Linco-Nichols</td>
<td>RIA</td>
<td>2</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>76</td>
<td>&lt; 0.1</td>
<td>100</td>
<td>ND</td>
<td>5-15</td>
<td></td>
</tr>
<tr>
<td>INSIK-5 DiaSorin</td>
<td>RIA</td>
<td>2.5</td>
<td>80</td>
<td>67</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>ND</td>
<td>5-25</td>
<td></td>
</tr>
<tr>
<td>INSULIN-CT CIS Bio International</td>
<td>RIA</td>
<td>3</td>
<td>60</td>
<td>43</td>
<td>60</td>
<td>119*</td>
<td>89.5</td>
<td>100</td>
<td>4.3-19.9 n = 24</td>
<td></td>
</tr>
<tr>
<td>Phadeseph Insulin RIA 100 Pharmacia &amp; Upjohn</td>
<td>RIA</td>
<td>2</td>
<td>60</td>
<td>46</td>
<td>75</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;20</td>
<td></td>
</tr>
<tr>
<td>Insulin-IRMA Medgenix-Biosource Europe</td>
<td>IRMA</td>
<td>1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>100</td>
<td>&lt; 0.1</td>
<td>162</td>
<td>&lt; 0.1</td>
<td>2-25 Subjects with normal OGTT</td>
<td></td>
</tr>
<tr>
<td>IMX-Insulin Abbott</td>
<td>IEMA</td>
<td>1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>77</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.8 ± 4.6 m ± 2 SD, n = 45</td>
<td></td>
</tr>
<tr>
<td>Bi-Insulin IRMA SANOFI-Pasteur Diagnostics</td>
<td>IRMA</td>
<td>0.5</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>75</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>75</td>
<td>2-17 n = 68</td>
<td></td>
</tr>
<tr>
<td>ACCESS Ultrasensitive Insulin SANOFI-Pasteur Diagnostics</td>
<td>IRMA</td>
<td>0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>75</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1-15</td>
<td></td>
</tr>
<tr>
<td>AIA-PACK IRI TOSOH-Eurogenetics</td>
<td>IEMA</td>
<td>2*</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt; 24.5</td>
<td></td>
</tr>
<tr>
<td>Auto DELFIA Insulin kit EGG WALLAC</td>
<td>IFMA</td>
<td>0.7</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>76</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.3-26.4 2.5-97.5th percentile</td>
<td></td>
</tr>
<tr>
<td>DAKO Insulin DAKO</td>
<td>IEMA</td>
<td>1.5</td>
<td>0.3</td>
<td>0.5</td>
<td>66</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.5-15.6 m ± 5 SD n = 80</td>
<td></td>
</tr>
<tr>
<td>SR1 Insulin BioChem Immunosystèmes</td>
<td>IEMA</td>
<td>2.5</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>38.6</td>
<td>ND</td>
<td>ND</td>
<td>4-23.5 5-95th percentiles</td>
<td></td>
</tr>
<tr>
<td>DPC</td>
<td>IEMA</td>
<td>2</td>
<td>&lt; 0.1*</td>
<td>ND</td>
<td>50</td>
<td>&lt; 0.1</td>
<td>ND</td>
<td>60</td>
<td>6-27 m ± 2 SD n = 57</td>
<td></td>
</tr>
</tbody>
</table>

D.L.: limit of detection (calculated with the profile of precision), hPI: human proinsulin, ND: not determined, * data given by the manufacturer. split proinsulins and rat insulin are gift of Eli Lilly.
The presence of anti-insulin antibodies can lead to spurious results in RIAs and two-site assays [40-42]. Many diabetic patients treated with human or animal insulin develop anti-insulin antibodies [43-47]. The prevalence of antibodies in patients who have only used human insulin is around 50% [44-47]. Anti-insulin autoantibodies have also been described in around 30% of Type 1 diabetic patients before insulin administration, as well as in insulin autoimmune hypoglycaemic syndrome and 1% of blood donors [45-51]. The presence of these circulating antibodies means that free plasma insulin (not bound to antibodies and biologically active) must be distinguished from antibody-insulin complexes. The nature of the interference by anti-insulin antibodies in RIA depends on their affinity and the method used to separate bound from free radioligand, thus leading to falsely high or low insulinaemia. In two-site immunoassays, anti-insulin antibodies can cause overestimation of plasma free insulin. The degree of overestimation depends on the comparative affinity of the (auto)antibodies and of the antibodies used in the assay. If the affinity of the assay antibodies exceeds that of the autoantibodies, displacement of the autoantibody-insulin complex may occur, leading to overestimation of the free insulin concentration [41].

Measurement of free insulin requires, first of all, the removal of antibodies and antibody-bound insulin. Total insulin (free + bound) can be assayed by dissociating bound insulin by acidification before the antibodies are separated from the reaction mixture. Most methods use polyethylene glycol (PEG), which precipitates the antibodies [52-57]. After centrifugation, free (or total) insulin is assayed in the supernatant. To obtain accurate results, PEG precipitation of antibodies must be performed on fresh serum/plasma immediately after collection [55, 56].

In two-site assays, other types of antibody may lead to falsely elevated results. These include anti-mouse antibodies (if mouse monoclonal antibodies are used) and autoantibodies such as rheumatoid factor [58-60]. These types of interference are not specific to insulin but to all two-site assays; RIA is not affected by these types of antibodies.

**EVALUATION OF INSULIN SECRETION: PROBLEMS AND PITFALLS**

Because insulin and C-peptide are secreted from β cells in equimolar amounts, their measurement should theoretically be interchangeable and give the same information on pancreatic secretion. However, owing to their different metabolism and the analytical characteristics of immunoassays, their plasma concentrations are not always equivalent in terms of pancreatic secretion and can lead to erroneous interpretation in numerous situations.

**Insulin and C-peptide**

In the absence of hepatic or renal insufficiency, insulin injection, circulating anti-insulin antibodies or haemolysis, the measurement of insulinaemia gives the same information as the plasma C-peptide assay. These two markers of β-cell secretory function are therefore interchangeable. The determination of insulin is often preferred because C-peptide immunoassays are generally less accurate and costlier, and very few reliable reference values are available in the literature. Moreover, plasma C-peptide is thermally unstable (around 2-3 weeks at -20°C and up to 6 months at -80°C), while plasma insulin can be stored for 3 days at 20°C, two weeks at 4°C and several months at -20°C [61-63]. In case of haemolysis, C-peptide is not degraded by IDE and can therefore be assayed instead of insulin [37]. In the presence of anti-insulin antibodies, free insulin must be determined or C-peptide assayed instead. However, anti-insulin antibodies bind proinsulin via its insulin moiety and greatly retard its clearance from the circulation. Because of cross-reaction with proinsulin in most C-peptide immunoassays, proinsulin bound to anti-(pro)insulin antibodies can lead to analytical interference and must be removed [54, 57]. In case of insulin injection (treatment of diabetes or surreptitious injections), it is not possible to distinguish between the insulin secreted by β cells and injected insulin (nearly all commercial insulins are now human). Plasma C-peptide can therefore be assayed to evaluate pancreatic secretion [64, 65]. In liver insufficiency, insulin metabolism is also impaired, which means that an abnormally large proportion of insulin reaches the peripheral circulation [66, 67]. Thus, the determination of insulinaemia can lead to overestimation of pancreatic secretion when plasma insulin values are compared to reference ranges in controls. The C-peptide/insulin molar ratio can be considered as an estimation of hepatic clearance. However, its use is limited by the lack of reference values and the complexity of the relationship between C-peptide and insulin. Indeed, due to their particular metabolic clearance rates, different concentrations of insulin may correspond to the same C-peptide concentration, depending on the sampling conditions [9]. As C-peptide is eliminated by the kidneys, values measured in patients with renal impairment are not interpretable [8]. When the glomerular filtration rate has fallen to less than 15-20 ml/min, the metabolism rate of insulin also declines. Moreover, impaired degradation of insulin due to uraemic toxins occurs in liver and muscles, which contributes to the prolonged half-life of insulin [68].
Specific and non-specific insulin immunoassays

The use of non-specific RIA leads to falsely high insulinaemia when proinsulinaemia is increased, as in IGT [11, 69, 70], Type 1 and 2 diabetes [11, 30, 34, 70-78], insulinoma [79, 80], familial hyperproinsulinaemia [79], insulin resistance [81], and uraemia [7]. Thus, in case of suspected hyperproinsulinaemia, a specific insulin assay must be used to assess β-cell insulinaemia. Moreover, when fasting plasma insulinaemia values measured by non-specific RIA exceed 50-100 mIU/l, especially in normoglycaemic patients with no known factors of insulin resistance (obesity, polycystic ovaries, hypercortisolism, etc.), samples should be re-tested with a specific assay and associated with a proinsulin assay in order to detect the possible presence of abnormal insulins or proinsulins which cross-react with non-specific assays. Most cases of familial hyperproinsulinaemia and abnormal insulins have been discovered by the investigation of subjects (generally tested in epidemiological studies) with high fasting plasma insulin assayed by RIA and normal insulin sensitivity [82-84]. However, non-specific methods can be useful in some situations, such as measurement of animal or human analog or abnormal insulins. To our knowledge, the cross-reactivity of specific insulin assays with abnormal insulins has not been reported.

INSULIN REFERENCE VALUES AND THEIR INTERPRETATION

To establish reference values and limits, insulinaemia must be measured in normoglycaemic subjects [85-88] with a normal body weight, as obesity is associated with insulin resistance [11, 18, 78, 89-91]. Moreover, as insulinaemia is most often measured during stimulation tests, reference values must also be determined for the most common tests, such as the OGTT and the intravenous glucose tolerance test (IVGTT). Given the diversity of assay methods, and the non-standardisation of stimulation tests until relatively recently (1979-1980 for OGTT [85, 86] and 1992 for IVGTT [92]), as well as the small number of studies with control populations large enough for meaningful statistical analysis [93, 94], few reliable reference values are available in the literature.

In fasting state

In adults, the studies by Boyns et al. [95] and Bonora et al. [96] involving large populations yielded insulinaemia values (RIA) of 3-32 and 3-26 mIU/l (mean ± 2 SD) respectively. Other studies gave similar or slightly higher values, the latter probably resulting from the use of less reliable assays and/or smaller samples [69, 97-101]. The reference values supplied with commercial assay kits are difficult to interpret as they are based on small numbers of subjects for whom little clinical information is given (Tables I and II). For most current RIA methods, the upper limit of the normal range is between 20 and 26 mIU/l, while the lower cut-off is often close to the detection limit, i.e. around 2 mIU/l.

The insulin values given by most IRMA/IEMA methods are generally lower than corresponding RIA values, but only a few studies have involved large population samples. Published fasting insulin plasma concentrations in a small sample of normal subjects were 3.3 ± 0.6 mIU/l (mean ± SEM, in 8 non-diabetic, non-obese men aged 22-36 years) [12], between 0.7 and 10 mIU/l (in 30 otherwise undefined “controls”) [31], and <1.8-10 mIU/l (in 20 healthy subjects with normal fasting plasma glucose, aged 23-47 years) [33]. Reference values obtained with commercial assays were generally similar (Table II). In a study of 207 normoglycaemic non-obese young adults, the reported fasting insulinaemia was ≤ 11 mIU/l (range) [102]. Thus, taken together, published data converge towards an upper limit of 11 mIU/l and a lower limit close to the detection limit, i.e. around 0.5-1 mIU/l.

The fasting plasma insulin concentration can be used to estimate insulin sensitivity, which is often diminished in obesity, IGT, Type 2 diabetes, uraemia, etc. [4, 18, 78, 89-91, 103, 104]. Although the euglycaemic hyperinsulinaemic clamp has come to be regarded as the gold standard, this accurate and precise technique is very complex and inapplicable to large populations; its use is reserved for specialised investigative centres and studies [105, 106]. This test requires the measurement of insulin to check the degree of induced hyperinsulinaemia. The IVGTT, conducted according to the Bergmann minimal model, is simpler but nevertheless requires from 15 to 28 samples according to the protocol, plus administration of insulin or tolbutamide at the 26th minute (a mathematical model allowing the calculation of different indices) [106, 107].

In a routine setting, plasma glucose and insulin values are only available in the fasting state or during an OGTT or IVGTT. Several studies have shown that fasting insulinaemia correlates with insulin sensitivity, especially when glycaemia is normal [108-111]. A better estimate of insulin sensitivity can be obtained (especially in case of glucose intolerance or Type 2 diabetes) by calculating the basal insulin/glucose ratio or using the HOMA index [104, 112-116]. Homeostasis model assessment (HOMA) is based on a computer-solved model of insulin: glucose interactions that plots an array of fasting plasma glucose concentrations likely to exist in subjects with various degrees of β-cell deficiency and insulin resistance [112]. The non-linearity of the model precludes an exact algebraic solution, but estimation is possible by using approximation: the HOMAIR (insulin resistance) index is calculated with the formula (fasting insulinaemia x glycaemia/22.5), insulinaemia being the
mean of three separate samples drawn 5 min apart (to avoid the confounding effects of oscillatory release). One of the major drawbacks of these indexes and basal insulinaemia is their poor day-to-day reproducibility, with coefficients of variation of up to 30% \[112, 115-119\]. They are therefore more adapted to epidemiological studies involving large populations than to individual assessments.

**During an OGTT**

For this test, the WHO report refers only to fasting and two-hour glycaemia, but generally the intermediate glucose values at 30, 60 and 90 min (and sometimes beyond 120 min) are also measured \[85\]. Reference values for this test are very rare, particularly with IRMA. Values at 30 min (generally corresponding to the usual secretion peak) are indicated in (Table II), together with those at 120 min. Values are quite different from one study to another, even when similar

Table II. Insulinemia (m IV/l) in nonobese normoglycemic adults.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ref</th>
<th>T0</th>
<th>T30</th>
<th>T120</th>
<th>n (M/W)</th>
<th>Age (Years)</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA 89c</td>
<td></td>
<td>19 ± 1</td>
<td>95 ± 10</td>
<td>76 ± 14</td>
<td>19(W)</td>
<td>19-49</td>
<td>40 g/m²</td>
</tr>
<tr>
<td>RIA 95a/b</td>
<td></td>
<td>15 (3-31)</td>
<td>72.8 (18-253)</td>
<td>20.4 (6-74)</td>
<td>109(M)</td>
<td>16-74</td>
<td>50 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.5 (2-60)</td>
<td>83.6 (9-396)</td>
<td>28.5 (4-168)**</td>
<td>111(W)</td>
<td>15-63</td>
<td>50 g</td>
</tr>
<tr>
<td>RIA 96d</td>
<td></td>
<td>14.7 ±11.6</td>
<td>ND</td>
<td>40 ± 50</td>
<td>134/113</td>
<td>10-69</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA 97d</td>
<td></td>
<td>23.7 ±14.8</td>
<td>162.9±131</td>
<td>53.3 ±61.6</td>
<td>10/2</td>
<td>19-67</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA 98f</td>
<td></td>
<td>23</td>
<td>87 ±5</td>
<td>62 ±5</td>
<td>13/8</td>
<td>24-59</td>
<td>40 g/m²</td>
</tr>
<tr>
<td>RIA 99c</td>
<td></td>
<td>10</td>
<td>100 ±7.5</td>
<td>80 ±10</td>
<td>48</td>
<td>18-25</td>
<td>1.75 kg/IBW</td>
</tr>
<tr>
<td>RIA 100</td>
<td></td>
<td>12.5 ±2.5</td>
<td>72.5 ±5</td>
<td>62.5 ±5</td>
<td>83 (M)</td>
<td>35 ±1.3</td>
<td>100 g</td>
</tr>
<tr>
<td>RIA 101h/d</td>
<td></td>
<td>12.7</td>
<td>90.2</td>
<td>54.2</td>
<td>51/39</td>
<td>42 ±22</td>
<td>75 g</td>
</tr>
<tr>
<td>IRMA 102a,g</td>
<td></td>
<td>&lt;11</td>
<td>13-110</td>
<td>4-53/12-5-72**</td>
<td>91/116</td>
<td>16-7-24.1</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA 118f</td>
<td></td>
<td>5.2 ±0.2</td>
<td>108/65</td>
<td>44.7 ±2.6</td>
<td>75 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA 165c</td>
<td></td>
<td>9 ±0.07</td>
<td>84 ±15</td>
<td>65 ±11</td>
<td>7/20</td>
<td>23-58</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 ±0.1*</td>
<td>125 ±15*</td>
<td>145 ±25*</td>
<td>11/6</td>
<td>60-82</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA 166b/f</td>
<td></td>
<td>5-14</td>
<td>33 ±5</td>
<td>10 (M)</td>
<td>23-29</td>
<td>75 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-12</td>
<td>29 ±4</td>
<td>17 (M)</td>
<td>60-80</td>
<td>75 g</td>
<td></td>
</tr>
<tr>
<td>RIA 168c</td>
<td></td>
<td>15 ±2.5</td>
<td>97.5 ±7.5</td>
<td>72.5 ±5</td>
<td>47 (M)</td>
<td>20-40</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 ±3</td>
<td>97.5±7.5</td>
<td>87.5±9</td>
<td>66 (M)</td>
<td>40-69</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ±2</td>
<td>87.5±10*</td>
<td>62.5±7.5*</td>
<td>43 (M)</td>
<td>40-69</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 ±2.5</td>
<td>82.5 ±8</td>
<td>80 ±8</td>
<td>26 (W)</td>
<td>20-40</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 ±2.5</td>
<td>90 ±8</td>
<td>80 ±8</td>
<td>30 (W)</td>
<td>40-69</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA 169a/b/d</td>
<td></td>
<td>6</td>
<td>36</td>
<td>30</td>
<td>22/16</td>
<td>40-64</td>
<td>75 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>43</td>
<td>30</td>
<td>7/10</td>
<td>65-92</td>
<td>75 g</td>
</tr>
<tr>
<td>RIA Coat-A-Counta</td>
<td>Insulin, DPC</td>
<td>3-35</td>
<td>35-220</td>
<td>15-180</td>
<td>22</td>
<td>ND</td>
<td>75 g</td>
</tr>
<tr>
<td>IEMA Abbott</td>
<td>AxSYM d</td>
<td>4.8 ±4.6</td>
<td>42.4±46.1</td>
<td>19.2±21</td>
<td>45</td>
<td>ND</td>
<td>75 g</td>
</tr>
<tr>
<td>IRMA Medgenixd</td>
<td></td>
<td>8.3 ±5</td>
<td>50.5±37.5</td>
<td>34.7±37.3</td>
<td>21(W)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.6±6.7</td>
<td>61.5±61.3</td>
<td>51.3±51.6</td>
<td>13(M)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a: mean, b: range, c: mean ± s.e.m., d: mean ± 2SD, e: median, f: mean ± se, g: 2.5-97.5 percentiles, h: geometric mean
* different between group age at same point time, ** different from male and female
1: office workers, 2: heavy physical laborers
assays are used. Values also vary markedly among individuals, especially at 30 min, when normal levels can differ by a factor of 10 or more (Table II). Insulinaemia values after a glucose load reflect the secretory capacity of pancreatic β cells. The best measure of insulin secretion is the ratio of the 30-min increment in the insulin concentration to the 30-min increment in the glucose concentration (insulinogenic index) following an oral glucose load in both normal and glucose-intolerant subjects [111, 114, 118, 120-122]. As this index becomes difficult to interpret if the denominator is very small or negative, an alternative index, defined as the ratio of the 30-min increment in the insulin concentration to 30-min glycaemia, has also been proposed [123]. These two indexes have good reproducibility and correlate with the first phase of insulin release following intravenous glucose infusion, but not with insulin resistance. A low index value is associated with altered glucose tolerance and increased risk of developing Type 2 diabetes in patients with IGT [101, 111, 114, 118, 120-122]. The 120-min plasma insulin value also correlates with the first phase of insulin release and insulin sensitivity [109-111]. The correlation between the 120-min plasma insulin concentration and insulin sensitivity is lower than the corresponding correlation with fasting insulin, and is found in subjects with normo- or impaired glucose tolerance, but not in Type 2 diabetic patients [110]. In patients with IGT, a high fasting and low 120-min insulinaemia are predictive of conversion to Type 2 diabetes [120, 124-126]. However, the reproducibility of the 120-min plasma insulin value is even poorer than that of fasting insulinaemia, limiting its interpretation [115, 117]. Factors affecting the absorption rate of glucose, such as gastrectomy, must also be taken into account [121].

During an IVGTT

The IVGTT is a standardised test which involves infusion of a 25% glucose solution for 3 min ± 15 s after a fast of between 10 and 16 hours; the glucose dose is 0.5 g/kg (maximum 35 g). Two baseline samples are taken 5 min apart, followed by samples 1, 3, 5 and 10 min after the end of infusion (time 0 corresponds to the end of the infusion) [92]. The first-phase insulin response (FPiR) is measured by adding the insulin concentration at t+1 min and t+3 min. FPIR is considered normal when the calculated insulinaemia (non-specific RIA) at t+1 min and t+3 min is < 25 (3rd percentile = 127) = 46 (1st percentile in controls, age range 8-77 years, 48 mIU/l (1st percentile in controls, age range 8-77 years = 129), 50 mIU/l (lowest response in controls, age range 5-45 years, glucose load 0.3 g/kg-130). Children have lower FPIR than adults: the 5th percentile is 18 mIU/l below 5 years of age and around 30 mIU/l in older subjects and adolescents [131]. Individual responses vary greatly at all ages [127-131].

The IVGTT can be used for early diagnosis of altered insulin secretion. The FPIR correlates with pancreatic insulin content and β-cell mass, and its reduction is one of the earliest detectable abnormalities in both types of diabetes [50, 77, 121, 122, 127-134]. The FPIR is also closely correlated with the insulinogenic index [111, 122, 134]. Contradictory results have been reported for the reproducibility of the FPIR, with coefficients of variation of <10% to 36% (range 11%-110%) [135, 136]. Thus, a low FPIR must be confirmed by a second test. FPIR is mainly used to predict the risk of progression to Type 1 diabetes in relatives of Type 1 diabetic patients, in conjunction with the determination of circulating autoantibodies and HLA [50, 127-131]. An FPIR < 25 mIU/l [137] or below the 1st percentile [129] is frequently associated with the development of Type 1 diabetes within 1 or 2 years in ICA+ (anti-islet cell antibody positivity) relatives of Type 1 diabetic patients. Analysis of the ICARUS (ICA Register Study) data set shows that a loss of FPIR (< 50 mIU/l) is associated with a 70% risk of developing Type 1 diabetes within five years in ICA+ first-degree relatives of diabetic patients, whereas the risk is only 17% when the FPIR is >100 mIU/l [50]. However, the risk is influenced by age and the ICA titre, reaching 90% in children under 10 years of age and 60% in adults over 40; 87% when ICA titres are high [80 Juvenile Diabetes Fundations Units: JDF-U]; and 73% when ICA titres are between 5 and 79 JDF-U. The sensitivity and specificity of two or more low FPIR values for predicting progression to Type 1 diabetes have been estimated at 60 and 96% respectively in first-degree relatives (age 2-29 years) of diabetic patients [131]. The highest predictive value (75%) is given by the combination of ICA+ (anti-islet cell antibody positivity), a consistently low insulin response, and HLA-DR3/4. However, the time of diabetes onset cannot be predicted from the decline in FPIR. Thus the FPIR measurement can only be used for screening purposes in conjunction with other parameters, and its predictive value should not be extended to non-relatives of Type 1 diabetic patients [129-131].

The loss of FPIR is not specific to Type 1 diabetes. Type 2 diabetic patients also have a low FPIR, and a low FPIR is also a predictor of progression to diabetes in glucose-intolerant subjects [77, 121, 122, 131, 134].

During a fasting test

Spontaneous hypoglycaemia, a disorder which can be caused by hyperinsulinism, insulinoma, insulin autoimmune syndrome and non-insulin-mediated factors, is the only clinical indication for the measurement of plasma insulin. Because symptoms of hypoglycaemia are non-specific, it is necessary to verify a low plasma glucose level when spontaneous symptoms occur and to demonstrate that symptoms are relieved through correction of the low glucose
level (Whipple’s triad) [138]. Inappropriate insulin secretion is rare, but hyperinsulinism is the most common cause of recurrent hypoglycaemia in neonates [139-141]. Although hypoglycaemia is a recognised cause of neurological sequelae, there is no unanimous accepted definition of the lower limit of normal glycaemia [142-144]. Generally, hypoglycaemia is defined by a plasma glucose concentration < 3.3-2.8 mmol/l in children and neonates [140-146]. These values are in part based on studies of neurological dysfunction and counterregulatory hormone secretion during hypoglycaemia [147, 148]. The measurement of glycaemia is often impossible when spontaneous symptoms occur, and the diagnosis is frequently made during a fasting test.

The supervised 72-hour fast is the classical diagnostic test for hypoglycaemia. It should be conducted in a hospital according to standardised procedures, especially in neonates or children for whom the duration of the fast is reduced and monitoring of glycaemia is more frequent [139].

As for hypoglycaemia, universally accepted criteria for relative hyperinsulinaemia have not been established for the diagnosis of insulinoma or hyperinsulinism. If hypoglycaemia occurs during the test and is associated with symptoms or signs, a concomitant insulinoma (non-specific RIA) > 5 mIU/l [145, 149], ≥ 6 mIU/l [139], > 10 mIU/l [4, 141] indicates inappropriate secretion and is a criterion for insulin media-
tion of hypoglycaemia. With a specific assay, plasma insulin > 2 mIU/l has been reported to be a critical criterion [140], and a preliminary study reported by an author using an RIA insulin value ≥ 5 mIU/l has suggested that hyperinsulinaemia may correspond to values ≥ 3 mIU/l [139]. Ratios of glucose to insulin (and vice-versa) are sometimes used to define hyperinsulinism/insulinoma [glucose (mg/dl)/insulin mIU/l] > 40, Turner ratio: insulin [mIU/l x 100]/glucose (mg/dl) -30 < 50, but seem to have little diagnostic value [139].

The plasma C-peptide assay is often associated in order to rule out factitious hypoglycaemia due to insulin administration or analytical interference [139, 140, 145, 150]. Some insulins may secrete much, or occasionally all, of their insulin-like activity as proin-
sulins [79, 80]. With specific insulin assays, such tumours would go undetected if insulin alone were measured. For this reason, some authors recommend measuring proinsulin in addition to insulin and C-peptide [139, 145]. Criteria for hyperinsulinism are levels of 0.2 nmol/l or 0.3 nmol/l for plasma C-peptide [139, 145, 150] and 5 pmol/l [80, 139] or 20 pmol/l [145] for proinsulinaemia, when hypoglycaemia is present. Patients with insulinomas or hyperinsulinism have insulin concentrations that rarely exceed 100 mIU/l [139, 140]. The contrast between high insulinaemia and low C-peptide (< 0.2 pmol/l) and/or pro-
insulin values in a hypoglycaemic patient is suggestive of recent insulin injection (plasma insulin values ranging from 200 mIU/l to 54,000 mIU/l have been observed) or points to analytical interference [139, 140, 151]. The insulin autoimmune syndrome (Hirata’s disease) is characterised by the combination of fasting hypoglycaemia, hyperinsulinaemia and insulin autoantibodies generally at a high level [42, 51, 139, 140]. This syndrome is a common form of hypoglycaemia in Japan, but seems very rare in other countries. Hypoglycaemia in these cases is linked to the buffering effect of the antibodies. As soon as insulin is secreted, it is bound to circulating antibodies, but also rapidly released. Therefore, continuous on-and-off binding occurs, that will depend upon the removal of insulin through a higher affinity interaction, e.g. with its receptor [51, 152]. When such a syndrome is suspected, insulin antibodies and free insulin (biologi-
cally active) must be determined, as the interference of antibodies in insulin assays is unpredictable [40-42].

Patients with hyperinsulinism usually do not have dramatically high insulin levels and sometimes cannot be distinguished from normal subjects by currently available insulin assays [139, 140]. Thus, plasma β-OH-butyrates, free fatty acids and the response of plasma glucose to glucagon, which are considered to be insulin surrogates, are also often determined at the end of the fast or during a hypoglycaemic disorder in order to improve the accuracy of the diagnosis [139, 140, 145, 146, 153].

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**PHYSIOLOGICAL VARIATIONS OF INSULIN VALUES**

**Influence of age**

Children have lower plasma insulin concentrations than adults, especially during stimulation tests, reflecting their higher sensitivity to insulin (Table III). Fasting insulinaemia in children under 9 years of age is about half or one-third that in adults and adolescents [154-160]. During an OGTT, infants (< 5-8 years depending on the study) have 30-min insulinaemia values about 2.3 times lower than children aged 11-12 years; between 6 and 12 years the difference is still about 40% [155-157]. During an IVGTT, young children (age < 5 years) have first-phase secretion about half that of children aged 6-10 years [131].

Sensitivity to insulin seems to decrease gradually during childhood and more rapidly during adolescence, before reaching adult values [155-163]. A decrease in glucose tolerance and an increase in insulin resistance have both been reported with ageing [164, 165]. However, some studies taking into account certain factors associated with age, such as the increase in fat mass, the decrease in physical activity and changes in diet, have attenuated these conclusions.
### Table III. Insulinemia in nonobese normoglycemic children.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ref.</th>
<th>T0</th>
<th>T30</th>
<th>T120</th>
<th>Age (years)</th>
<th>Glucose</th>
<th>n (M/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>154f</td>
<td>7±1</td>
<td>15±3</td>
<td>45±9</td>
<td>&lt;24 h</td>
<td>2 g/kg</td>
<td>11</td>
</tr>
<tr>
<td>RIA</td>
<td>155c</td>
<td>10±3*</td>
<td>48±5*</td>
<td>30±4*</td>
<td>1.5-5.7</td>
<td>1.75 g/kg</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16±3*</td>
<td>80±8*</td>
<td>50±4*</td>
<td>5.8-10.9</td>
<td>75 g max</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20±6*</td>
<td>114±12*</td>
<td>80±10*</td>
<td>11-18</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>RIA</td>
<td>156c</td>
<td>5.8±2.3</td>
<td>27.4±4*</td>
<td>16.1±3.8</td>
<td>0.3-5</td>
<td>30 g/m2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6±2.4</td>
<td>46.5±5.4*</td>
<td>16.9±4.5</td>
<td>6-10</td>
<td>30 g/m2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.3±3.7</td>
<td>64±6.9*</td>
<td>29±4.6*</td>
<td>11-15</td>
<td>30 g/m2</td>
<td>33</td>
</tr>
<tr>
<td>RIA</td>
<td>157f</td>
<td>11.7±1.2</td>
<td>55.9±9.4</td>
<td>40.1±3.9</td>
<td>2-13</td>
<td>1.75 g/kg</td>
<td>16/7</td>
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<tr>
<td></td>
<td></td>
<td>15±1*</td>
<td>82±8.4*</td>
<td>82.6±8.9*</td>
<td>13-20</td>
<td>100 g max</td>
<td>23/17</td>
</tr>
<tr>
<td>RIA</td>
<td>171a</td>
<td>8</td>
<td>56</td>
<td>30/44**</td>
<td>5-10</td>
<td>1.75 g/kg</td>
<td>16/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10/15**</td>
<td>65/60</td>
<td>45/55**</td>
<td>11-15</td>
<td>100 g max</td>
<td>23/21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>72/65</td>
<td>52/60</td>
<td>16-29</td>
<td>39/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>54</td>
<td>65</td>
<td>&gt;30</td>
<td>54/59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>69e/b</td>
<td>16(11-24)</td>
<td>59.5(33-390)</td>
<td>33.5(22.6-91)</td>
<td>10-16</td>
<td>1.75 g/kg</td>
<td>11/9</td>
</tr>
<tr>
<td>RIA</td>
<td>160d</td>
<td>4.2±6.2/4.6±5.6</td>
<td>3</td>
<td>275/265</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.9±6.4/6.5±6.8**</td>
<td>6</td>
<td>272/290</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5±7.4/8.5±8.8**</td>
<td>9</td>
<td>314/311</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10±10.2/13.7±13.4**</td>
<td>12</td>
<td>314/327</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.1±10.6/14.8±11.4**</td>
<td>15</td>
<td>283/308</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3±10/13.3±10.8**</td>
<td>18</td>
<td>251/276</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RIA</td>
<td>161e(b)/d</td>
<td>7.7 (2.6-16)</td>
<td>1</td>
<td>9.1±3.6</td>
<td>11/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11* (8.2-22)</td>
<td>2+3</td>
<td>12.4±4.6</td>
<td>8/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13* (8.5-23)</td>
<td>4+5</td>
<td>15.2±2.6</td>
<td>7/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.2 (2.2-13)</td>
<td>adults</td>
<td>30±10.2</td>
<td>10/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRMA</td>
<td>160c</td>
<td>3±0.6*</td>
<td>0-8</td>
<td>17</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>12±2.2</td>
<td>8-16</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.2±3.2</td>
<td>&gt;16</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a: mean, b: range, c: mean ± s.e.m., d: mean ± 2SD, e: median, f: mean ± se
* different between group age at same point time, ** different from male and female.
Indeed, such studies have shown that insulin secretion during an OGTT in healthy non-obese elderly adults is close, if not identical, to values in young adults with similar physical activity and diet [95, 166-171].

Influence of gender

Fasting plasma insulin concentrations measured by RIA or IRMA are similar in both sexes [95, 96, 102, 157]. Some studies have shown higher values in females during adolescence, but this could be due to differences in the stage of puberty at a given chronological age [159, 171]. During stimulation, the difference appears more clear-cut, although it is not found in all studies. Increased insulin secretion during an OGTT has been reported at 60 or 120 min in females aged 5 to 30 years; beyond this age, insulinaemia (RIA) becomes similar again [171] or remains higher [95], depending on the study. A study of young adults (age 16.5-24 years) also showed higher insulinaemia (IRMA) in females, but only at 120 min of an OGTT [102]. These differences were not linked to the use of oral contraceptives [95, 102, 171]. In sum, it seems that females only have higher insulinaemia at 60 or 120 min after a glucose load, and especially in adolescence and young adulthood.

PATHOPHYSIOLOGICAL VARIATIONS AND CLINICAL INDICATIONS OF INSULINAEMIA ASSAYS

Interpretation of insulin assay results in terms of the reference values should provide a diagnosis, or at least a suspicion, of a variety of conditions, such as inappropriate secretion, insulin deficiency leading to diabetes mellitus, and hyperinsulinaemia linked to insulin resistance, whether associated with diabetes or not. It must be remembered that blood insulin values can only be interpreted in conjunction with blood glucose levels.

Clinical indications for insulin measurement are classically limited to the diagnosis of insulinoma and hyperinsulinism. IGT and diabetes are diagnosed solely on the basis of chronic hyperglycaemia [85-88]. Thus, measurement of plasma insulin has no clinical value in the diagnosis or management of IGT/diabetic patients, with the exception of a few cases, including the syndrome of severe insulin resistance and abnormalities in β-cell secretory products. Insulin measurements are also used in experimental investigations to study the pathophysiology of various disorders, especially diabetes and IGT.

Impaired glucose tolerance and diabetes

Type 1 diabetes – The measurement of insulin in Type 1 diabetes can be useful in particular situations such as pharmacological, pharmacokinetic and pathophysiological studies [45-47]. Most Type 1 diabetic patients develop insulin antibodies, which means that free insulin needs to be determined [41-48]. Total insulinaemia (free + bound) is rarely measured, as the biological effects of insulin are produced by its free form. However, their determination can be useful when investigating the pathophysiology of some rare diseases linked to anti-insulin antibodies. In these cases, and because antibodies and anti-insulin antibody complexes have also been implicated in various diabetic complications, their determination is often combined [45-47].

Anti-insulin antibodies rarely lead to therapeutic problems. The clinical implications of high insulin antibody titres include their effect on insulin kinetics, which may influence glycaemic control, lipoatrophy at the insulin injection site, and their ability to cross into the foetal circulation (whether pregnancies in diabetic mothers with antibodies are at an increased risk of serious or fatal complications is not clear, but the neonates of these mothers are probably at an increased risk of neonatal hypoglycaemia and macrosomia) [45, 46, 172]. Moderately elevated insulin antibody titres can lead to high postprandial glycaemia due to a delay in the initial rise in free insulin, and can also cause prolonged hypoglycaemia by the release of insulin from insulin-antibody complexes at inappropriate times [45, 173]. In very rare cases, high insulin antibody titres lead to immunological insulin resistance [43, 45, 47].

The main use of anti-insulin-(auto)antibody measurement is in determining susceptibility to Type 1 diabetes, in conjunction with other markers (low FPIR, and genetic and auto-immune markers) [46, 48, 50].

Type 2 diabetes – Type 2 diabetes and IGT are metabolically heterogeneous conditions [78, 174, 175]. Since the time when the first insulin assay revealed the presence of insulin in patients with Type 2 diabetes, insulin measurement has been central to the debate on whether the main defect causing hyperglycaemia is insulin resistance or insulin deficiency. It is generally accepted that Type 2 diabetic patients and some patients with IGT have β-cell dysfunction and insulin resistance, but the relative importance of these disorders is debated, and their role in the pathogenesis of Type 2 diabetes remains controversial. As hyperglycaemia itself can impair insulin secretion and cause insulin resistance, both of these abnormalities may be secondary to hyperglycaemia [78]. Recently, the expert committee on the diagnosis and classification of diabetes mellitus, convened by the American Diabetes Association, as well as a provisional report of WHO, defined Type 2 diabetes as resulting from insulin resistance with an insulin secretory defect (ranging from predominant insulin resistance with relative insulin
deficiency to predominant insulin secretory defect with insulin resistance) [87, 88].

The development of specific assays for insulin and proinsulins established that a high proportion of the insulin measured by non-specific RIA in Type 2 diabetes is indeed intact proinsulin and des 31,32 proinsulin [30, 34]. The proportion of proinsulin in Type 2 diabetic patients varies with the degree of glycaemia and obesity [11, 71, 72] and the treatment [71, 73-75], reaching 50% of the total insulin measured in plasma [34, 72, 75, 76]. If a poorly specific insulin RIA had been used in this setting, there would have been an overestimation of the insulin response, suggesting insulin resistance. Thus, studies using specific insulin assays have highlighted the relative insulin deficiency in Type 2 diabetes [30, 71, 72]. Moreover, in some Type 2 diabetic patients, the primary defect may be impaired insulin secretion, leading to absolute insulin deficiency and, secondarily, to insulin resistance [78, 118, 175-177]. Patients with this form of Type 2 diabetes are lean [172]. However, insulin resistance plays an important role in the development of most patients with Type 2 diabetes (particularly those who are obese) and is frequently present in the very early stages of glucose intolerance in subjects who go on to develop diabetes [18, 78, 101, 124-126]. In several populations, it has been shown that high fasting insulinemia associated with low insulin response to a glucose load are predictive of Type 2 diabetes in subjects with IGT. Therefore, it would seem that Type 2 diabetes occurs only when the pancreas is unable to compensate for insulin resistance by adequate secretion of insulin [78, 120, 124-126].

Syndromes of extreme insulin resistance – Type A and type B insulin resistance, leprechaunism, Rabson-Mendenhall syndrome and lipodystrophic diabetes are rare disorders characterised by severe or extreme/total insulin resistance [90, 178]. In contrast to the early onset of the type A syndrome, patients with type B insulin-resistance syndrome are middle-aged and, in addition to the common features of severe insulin resistance (acanthosis nigricans, etc.), are characterised by the presence of autoantibodies to the insulin receptor [90, 178]. These patients may also develop fasting hyperglycaemia or hypoglycaemia during the course of their disease, even subsequent to a period of diabetes [90, 178]. The Rabson-Mendenhall syndrome, a variant of type A, is characterised by dental dysplasia and other dysmorphic features [90, 178]. This very rare syndrome appears in childhood and generally has a poor prognosis. Lipodystrophy syndromes are characterised by severe triglyceridaemia leading to pancreatitis and cirrhosis. They are subclassified into the Berardinelli-Seip syndrome (congenital generalised lipodystrophy) and the Lawrence syndrome (acquired lipodystrophy) [90, 178, 181]. In all these syndromes, fasting plasma insulin values (non-specific RIA) vary greatly between subjects: from 30 to 300 mIU/l and up to 900 mIU/l after a glucose load [179-181]. Leprechaunism is a very rare congenital syndrome characterised by intrauterine growth retardation, lipodystrophy, facial dysmorphism and extreme insulin resistance. Few of these infants live beyond the first year of life, although some survive until adolescence [90, 178, 182-184]. In leprechaunism, fasting plasma insulin can reach 1,000 mIU/l, and more than 9,000 mIU/l after a glucose load [182-184].

Type A insulin resistance, Rabson-Mendenhall syndrome and leprechaunism resulting from genetically determined abnormalities of insulin action are associated with insulin-receptor mutations [90, 178]. Alteration of the insulin receptor has not been demonstrated in patients with lipodystrophic diabetes, but it is assumed that the lesion relates to post-receptor signal transduction [90, 178].

Mutation of the proinsulin gene

Familial hyperproinsulinaemia – Familial hyperproinsulinaemia, a very rare genetic syndrome inherited in an autosomal dominant pattern, is characterised by markedly increased levels of abnormal proinsulin or its conversion intermediates [79, 84, 185-192]. The use of non-specific RIAs which cross-react with these mutant proinsulins leads to falsely high insulinemia at levels encountered in extreme insulin resistance. The development of specific insulin and proinsulin assays, together with genetic studies, has identified four mutations in the proinsulin gene [and one mutation in the prohormone convertase 1 (PC 1) gene] associated with high plasma levels of proinsulins [192]. All subjects with familial hyperproinsulinaemia caused by a mutation in the proinsulin gene are heterozygous; they secrete normal insulin and mutant proinsulins. The first identified mutant, resulting from the substitution of an amino acid in the insulin B chain, is predominantly secreted from β cells via the unregulated pathway [185]. Despite intact AC and BC junctions (Fig. 1), this results in the secretion of unprocessed mutant proinsulin, probably due to a conformational change inhibiting the conversion of proinsulin to insulin [185]. The second and third mutations result in the substitution of leucine [84] or histidine [186-190] for arginine at position 65 of the proinsulin AC cleavage site, leading to the secretion of an abnormal proinsulin. These mutations, which seem to inter-
fere with normal enzymatic recognition, lead to a circulating proinsulin similar to the conversion intermediate cleaved only at the BC junction, i.e. des 31,32proinsulin [84, 186-190]. The His – 65 mutation has been encountered in six families (U.S.A., Japan and Germany). This hyperproinsulinaemia is associated with IGT. Type 2 diabetes or normal glucose tolerance (the three-generation Caucasian family is normoglycaemic) and seems to be the most common form of familial hyperproinsulinaemia. In the basal state and after a glucose load, normoglycaemic subjects have high proinsulin levels (around 300 pmol/l) when fasting (normal ≤ 16 pmol/l) and up to 2,500 pmol/l at 60 min of an OGTT, but intact insulinaemia is slightly reduced [190]. However, if insulin bioactivity is assumed to be around 10% of the activity for a mutant proinsulin (such as des 31,32proinsulin), the intact insulin response can be considered as normal. The fourth mutation leads to the substitution of a proline for the arginine at position 65 [191]. This mutation, encountered in one family, prevents cleavage at the AC junction and is not associated with diabetes.

It is not clear why subjects with the same mutant proinsulin gene can be normoglycaemic, IGT or diabetic. Insulin secretion by the normal gene may not be sufficient to maintain plasma glucose in the normal range, particularly in the presence of other diabetogenic factors, whether genetic or acquired (obesity, etc.)

A mutation affecting the PC 1 gene has been identified in obese women with abnormal glucose homoeostasis and adrenal function. Although not involving the proinsulin gene, this mutation, by preventing the processing of proPC1, causes its retention in the endoplasmic reticulum, leading to high but normal intact and des 64,65proinsulin levels and very low insulin and des31,32proinsulin levels. In addition to this abnormal proinsulin processing, there is evidence of impaired processing of proproinsulin and downstream degradation of insulin in liver and muscles [103, 199].

**Familial mutant insulin** – The synthesis and secretion of three structurally abnormal insulins has been identified in six families [82, 193-195]. Plasma extraction and structural analysis of the abnormal insulins identified amino-acid substitutions ([LeuA3]-, [SerB24]- and [LeuB25]-insulin, also designated as insulin Wakayama, Los Angeles, and Chicago respectively), leading to molecules with very low insulin bioactivity and prolonged half-lives (0.14% and 35 min for [LeuA3]-insulin, = 2% and 15 min for [LeuB25]-insulin) [82, 193]. The main characteristics of patients with mutant insulins are hyperinsulinaemia, normal insulin sensitivity, a reduced C-peptide: insulin molar ratio, and glucose tolerance varying from normal to diabetic. All subjects are heterozygous and secrete normal and mutant insulins at an equimolar ratio. Hyperinsulinaemia is usually marked, with fasting values > 80 mIU/l (normal + mutant, measured by non-specific RIA), associated with a low C-peptide: insulin molar ratio. The increased insulinaemia and diminished ratio have been ascribed to the markedly prolonged half-life of the insulin mutants: when insulin was extracted from the pancreas of a patient with mutant insulin, approximately 50% proved to be human (non-mutant) insulin [193].

The prevalence of genetic mutations in the proinsulin gene in the general population is unknown, but seems to be very low. However, detection of such abnormalities in asymptomatic persons raises the possibility that similar defects may occur more frequently than reported.

**Cirrhosis** – Insulin resistance is present in nearly all patients with cirrhosis [196]. Cirrhotic patients have elevated insulinaemia during fasting or after a glucose load [66-67, 121, 197, 198]. Fasting plasma insulin concentrations are increased two-fold [66] to six-fold [197] in comparison with control subjects. There is defective metabolism of insulin by the cirrhotic liver, so that an abnormally large proportion of insulin secreted by the pancreas reaches the peripheral circulation. The metabolic clearance of insulin in cirrhotic patients is reduced to one-half the rate found in controls [198]. The chronic hyperinsulinaemia resulting from the reduced clearance seems to cause insulin resistance, which can therefore be interpreted as an adaptive mechanism protecting against hypoglycaemia [67, 196].

**Uraemia** – When the glomerular filtration rate is < 15-20 ml/min, the metabolic clearance rate of insulin decreases rapidly [68, 103, 199]. Moreover, it seems that accumulation of dialysable uraemic toxins, with progressive loss of renal function, also inhibits the degradation of insulin in liver and muscles [103, 200, 201]. Uraemic patients are all insulin-resistant, and about half of them are glucose-intolerant [103, 202]. Varying plasma insulin concentrations following a glucose load have been reported in uraemic patients [103, 202]. Two distinct groups of uraemic patients may exist: one with normal glucose tolerance and increased insulinaemia and another with IGT and low or normal insulinaemia [103, 202]. Vitamin D seems to play an important role in the development of glucose intolerance in uraemic patients: low plasma 1,25(OH)2D3 is associated with inhibition of insulin secretion [103, 203]. Intravenous administration of 1,25(OH)2D3 to uraemic patients on haemodialysis leads to an increase in insulin secretion and a reversal of glucose intolerance [103, 203, 204]. This effect is independent of any change in serum parathormone concentration [103, 203].
CONCLUSION

In recent years, the introduction of specific monoclonal antibodies labelled with enzymes or fluorescent, chemiluminescent or isotopic markers has led to the development of two-site assays for human insulin. Some of these immunometric methods have good precision and specificity (no cross-reactivity with intact or des 31,32 proinsulins) and a low detection limit (< 1 m IU/l). Specific assays discriminate between plasma insulin and proinsulin, allowing better evaluation of pancreatic insulin secretion. In particular, studies using specific insulin assays have highlighted insulin deficiency in Type 2 diabetes. The interpretation of insulinaemia values can be erroneous if the clinical setting (cirrhosis, etc.) is not taken into account and if analytical interference (haemolysis, anti-insulin antibodies, etc.) is overlooked. Large-scale use of insulin assays remains a research tool rather than a clinical aid. Reference and cut-off values of plasma insulin are still need to be satisfactorily established, as values reported in the literature are divergent. There is a need to standardise the results and to improve comparability among studies.

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