References


Homocysteine enzymatic assay
A strong, independent risk factor for cardiovascular disease

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CH-6343 Rotkreuz
Switzerland
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With the cobas modular platform, the cobas 4000, 6000 analyzer series and cobas 8000 modular analyzer series, Roche has developed a platform concept based on a common architecture that delivers tailor-made solutions for diverse workload and testing requirements. The cobas modular platform is designed to reduce the complexity of laboratory operation and provide efficient and compatible solutions for network cooperation.

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- Multiple configurations with tailor-made solutions for higher efficiency and productivity
- Consolidation of clinical chemistry and immunochemistry with more than 200 parameters for cost and workflow improvements
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- Consistency of interaction with hardware, software and reagents for less training and more staff flexibility
- Consistency of patient results due to a universal reagent concept

Cardiovascular disease
Cardiovascular disease (CVD) is a major health concern that continues to grow. CVD is already responsible for more deaths globally than any other disease and the huge burden it places upon healthcare systems and society is predicted to become even greater:

- CVD was estimated to be responsible for 173 million deaths in 2008, which represents 30% of the global total. Of these deaths, an estimated 72 million were due to ischemic heart disease (IHD) and 5.7 million were due to cerebrovascular disease (stroke). 1
- More than 80% of CVD deaths occur in low- and middle-income countries, with men and women affected almost equally. 1
- Annual deaths from CVD are predicted to reach almost 25 million by 2030, with heart disease and stroke remaining the leading causes. 1
- The projected economic cost to the USA in 2010 was $442 billion, which takes into account the cost of health services, medication, and lost productivity. 1

Mitigating the impact of increasing CVD can be achieved by combining the early detection of at-risk individuals with the adoption of risk-lowering behaviors. The importance of reliable diagnostic markers for identifying at-risk individuals is highlighted by the fact that the first sign of heart disease in 25% of adults with CVD is fatal heart attack. 2 Furthermore, conventional risk factors, such as high serum cholesterol levels and high blood pressure, fail to account for all cases of CVD. For example, more than 75% of heart attacks occur in patients with normal serum cholesterol. 3 Therefore, there is a clinical need to expand the number of diagnostic tools available for evaluating an individual’s risk of CVD. Numerous extensive studies have demonstrated that the concentration of blood homocysteine, a thiol-containing amino acid, can serve as an excellent ‘new’, clinically useful risk factor for CVD. 4-10

Homocysteine as a causal factor in CVD
An association between elevated blood homocysteine (hyperhomocysteinemia) and atherothrombotic disease was first proposed in the late 1960s following observations in children with homocystinuria (a rare autosomal recessive disorder caused by enzyme deficiencies in homocysteine metabolism) who displayed extensive atherosclerotic plaques similar to those observed in adults with CVD. 11 Subsequent observations from approximately 80 clinical and epidemiologic studies have demonstrated that hyperhomocysteinemia is an independent, dose-dependent risk factor for atherosclerotic vascular disease and for arterial and venous thromboembolism. 6 For example, moderate-to-intermediate hyperhomocysteinemia is present in 12-47% of patients with coronary, cerebral, or peripheral arterial occlusive diseases. 12 Homocysteine is highly cytotoxic and elevated levels within the bloodstream are believed to damage the endothelial lining of arterial vessels, which subsequently leads to inflammation and the formation of atherosclerotic plaques that eventually restrict the flow of blood to the heart and other organs (Figure 1). Several other, potentially synergistic, mechanisms have been proposed to explain how excess homocysteine promotes atherosclerosis:
- Alteration of endothelial phenotype and reduced production of the endogenous vasodilator nitric oxide 12-19
- Deposition of cholesterol and other fats following degradation of dense aggregates formed from homocysteine thiolactone and low-density lipoprotein 20
- Connective tissue changes induced by exposure and proliferation of the underlying smooth muscle and extracellular matrix 19

Figure 1: Excess circulating homocysteine increases the risk of cardiovascular disease.
“Numerous clinical and epidemiologic studies have established elevated blood homocysteine as a potent independent risk factor for vascular disease in the general population.” 10
Measurement of homocysteine

Homocysteine is produced within cells by the metabolism of methionine from dietary protein. Intracellular concentrations are kept low by export into the plasma, where it becomes oxidized rapidly and circulates as one of three forms (Figure 2). The parameter measured most frequently in clinical laboratories is the combined sum of all three forms, which is referred to as “total homocysteine”.

**Homocysteine in risk assessment and diagnosis**

The American Association for Clinical Chemistry recommends measurement of blood total homocysteine in three clinical settings.  
- **Assessment as a risk factor for CVD**
- **Diagnosis of homocystinuria**
- **Identification of individuals with (or at risk of developing) folate (vitamin B<sub>9</sub>) or cobalamin (vitamin B<sub>12</sub>) deficiency**

Although homocysteine screening of the general population is currently not recommended, recommendations for screening regimens in the three clinical settings described above have been published (Table 3).

**Hyperhomocysteinemia** detected through testing can be classified as either moderate, intermediate or severe (Table 2).

**Terminology**

<table>
<thead>
<tr>
<th>Protein-bound</th>
<th>Non-protein-bound (free)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homocysteine</td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td></td>
</tr>
<tr>
<td>Homocysteine mixed disulphide</td>
<td></td>
</tr>
<tr>
<td>Homocysteine cysteine mixed disulphide</td>
<td></td>
</tr>
</tbody>
</table>

**Structure**

- Proportion of total: 70 – 90 %, 8 – 19 %, < 2 %

**Table 1: Upper reference limits for blood total homocysteine**

* Results from a European population (n = 800) not supplemented with folate. Total homocysteine 2 hours after methionine load is approximately 75 % of the value measured after 4 hours.

<table>
<thead>
<tr>
<th>Demographic group</th>
<th>Upper reference limit for total homocysteine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate supplemented</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>8</td>
</tr>
<tr>
<td>Children (&lt;15 years)</td>
<td>8</td>
</tr>
<tr>
<td>Adults (15 – 65 years)</td>
<td>12</td>
</tr>
<tr>
<td>Elderly (&gt;65 years)</td>
<td>18</td>
</tr>
<tr>
<td>Post-methionine load (4 – 6 hours)*</td>
<td>5-fold fasting level, or 40 µmol/L increase *</td>
</tr>
</tbody>
</table>

**Target group**

- CVD patients or patients at high risk of CVD
- Patients with symptoms of homocystinuria or a sibling with homocystinuria
- Patients with homocystinuria
- Patients with symptoms of or at risk of, folate or cobalamin deficiency
- Patients treated for folate or cobalamin deficiency

**Rationale for testing**

- Exclude homocystinuria and identify patients at high risk of CVD events and mortality
- Monitor treatment response and compliance
- Exclude or confirm homocystinuria
- Exclude or confirm deficiency
- Monitor treatment response or detect relapse

**Frequency of testing**

- At entry into medical system, possibly every 3 – 5 years thereafter
- Every 2 – 4 weeks until values are stable, then annually or following change in treatment regimen
- At entry into medical system; every 3 – 5 years thereafter
- 2 – 4 weeks after initiation of therapy, then annually or when symptoms arise

**Figure 3: Range of values for blood total homocysteine observed in individuals affected by different physiologic and pathologic factors**

* Combined with folate deficiency
** Effect of alcohol depends on whether consumption is high and chronic (increased total homocysteine) or moderate (decreased total homocysteine)

**Abbreviations**: CVD, cardiovascular disease.

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**Figure 2: The three forms of homocysteine present within the circulation**

Normal fasting levels of blood total homocysteine are considered to be 5 – 15 µmol/L, although European laboratories tend to use a value of 12 µmol/L as the cutoff value for normal fasting total homocysteine in adults. Upper reference limits differ depending on an individual’s age and whether they have access to food fortified with folate or dietary supplements (Table 1).

**Table 2: Classification of hyperhomocysteinemia**

<table>
<thead>
<tr>
<th>Category</th>
<th>Homocysteine level</th>
<th>Prevalence within the general population: &lt; 10 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate</td>
<td>15 – 30 µmol/L</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>30 – 100 µmol/L</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>&gt; 100 µmol/L</td>
<td>0.02 %</td>
</tr>
</tbody>
</table>

**Table 3: American Association of Clinical Chemistry recommendations for homocysteine testing**

**Table 4: For use with plasma total homocysteine, µ mol/L**

<table>
<thead>
<tr>
<th>Plasma total homocysteine (µmol/L)</th>
<th>C677T MTHFR*</th>
<th>CBS-deficiency</th>
<th>MTHFR-deficiency</th>
<th>Cbl mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
</tr>
<tr>
<td>10</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
</tr>
<tr>
<td>100</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
<td>0.02 %</td>
</tr>
</tbody>
</table>

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**Figure 4: Plasma total homocysteine (µmol/L)**

*Combined with folate deficiency
**Effect of alcohol depends on whether consumption is high and chronic (increased total homocysteine) or moderate (decreased total homocysteine)
Further evidence for the effect of homocysteine reduction on CVD risk comes from a large epidemiologic study of the impact of the folate fortification program in the USA and Canada. The fortification program began in 1996 as an attempt to prevent birth defects, but the study found the program also reduced the mortality rate from stroke and heart attacks. For example, stroke mortality declined almost 5% per year following fortification compared with a decline of only 1% prior to 1997. Overall, the researchers estimate the folate fortification program prevented 31,000 deaths from stroke and 17,000 deaths from heart disease every year from 1998 to 2001.

Clinical approach to lowering homocysteine
Patients with manifest CVD or at high risk of developing CVD should have their total homocysteine measured and be encouraged to adhere to their physician's advice for treatment if the level is >15 μmol/L. Total homocysteine levels can be lowered by various homocysteine-lowering agents, such as vitamin supplements, betaine, and N-acetylcysteine. Lifestyle changes can also help reduce levels and the adoption of healthy behaviors, such as a balanced diet, cessation of smoking, regular exercise, and consumption of only moderate amounts of caffeine and alcohol, all have considerable positive health benefits beyond the prevention of CVD (Figure 5).

Meta-analysis of 72 studies has demonstrated significant associations between blood total homocysteine and the risk of ischemic heart disease, deep vein thrombosis & pulmonary embolism, and stroke. According to the authors, the results of the meta-analysis provide further strong evidence for a causal relationship between elevated blood homocysteine and CVD. The authors estimate that lowering blood total homocysteine by 3 μmol/L would reduce an individual's risk of ischemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24% (Figure 4).

"Modest reduction of homocysteine is predicted to reduce the risk up to 25% for cardiovascular disease."
Homocysteine in non-CVD settings

Diagnostic testing of blood total homocysteine can be useful in many non-CVD clinical settings:

Homocystinuria
Rare genetic deficiencies in the enzymes responsible for homocysteine metabolism lead to severe hyperhomocysteinemia (usually >100 μmol/L) and urinary excretion of large amounts of homocysteine. Individuals with this metabolic disorder have high risk of premature, and frequently fatal, thromboembolic events. Children and young adults should be tested if they exhibit symptoms of thromboembolism, lens dislocation, progressive myopia, osteoporosis, Marfan-like appearance, unexplained mental retardation, psychiatric disorders, and megaloblastic anemia. Siblings or children of patients with homocystinuria should also be tested.

Folate and cobalamin deficiencies
Folate deficiency occurs in individuals of all ages and usually results from poor diet, malabsorption, alcoholism, or the use of certain drugs; it is also common during pregnancy. The prevalence of folate deficiency has decreased significantly in many non-CVD settings:

- In being cost-effective, fast, robust, easy to perform, stable over time with excellent accuracy and precision, and with an analytical range covering the 5 – 99.5 percentiles of the general population, the Roche Homocysteine enzymatic assay fulfills all the performance criteria recommended by the American Association for Clinical Chemistry.

Comparison with other methods
There are currently three main analytical methods for evaluating blood total homocysteine levels in patient samples:

- Chromatographic methods
- Immunoassays
- Enzyme cycling methods

There are significant differences between the three methods with regard to assay precision, speed, and cost (Figure 6). Enzyme cycling uses fewer reagents and is faster on a ‘per test’ basis, which means the method is also the least expensive. Additional savings are possible due to the absence of a need for sample pretreatment, specialized instruments, or dedicated operators.

Roche Homocysteine enzymatic assay

The Roche Homocysteine enzymatic assay incorporates a range of features to ensure ease of use and reliability of results. The fully automated assay is based on the enzyme cycling method and requires only 10 minutes to generate results from samples as small as 14 μL. The assay is as user-friendly as conventional clinical chemistry assays and is compatible with all automated clinical chemistry analyzers, including cobas c, COBAS INTEGRA, and MODULAR® ANALYTICS systems.

In being cost-effective, fast, robust, easy to perform, stable over time with excellent accuracy and precision, and with an analytical range covering the 5 – 99.5 percentiles of the general population, the Roche Homocysteine enzymatic assay fulfills all the performance criteria recommended by the American Association for Clinical Chemistry.

Cystathionine interference
The Roche Homocysteine enzymatic assay displays greater specificity than other methods due to a lack of interference from cystathionine (an intermediate product in homocysteine metabolism). Cystathionine levels are significantly elevated in millions of renal failure patients and methods which are affected by this interference can overestimate blood total homocysteine by as much as 20 – 300 %.

Chemical principle of the enzyme cycling method
The Enzyme cycling method represents the latest cutting-edge technology and has rapidly become the preferred method used in clinical laboratories, especially those routinely testing large numbers of samples. The Enzyme cycling method is based on the following chemical reactions:

1. Step: Oxidized Hcy that is bound to protein is first reduced to free Hcy. 2. Step: Hcy then reacts with a co-substrate, S-adenosylmethionine (SAM), to form methionine (Met) and S-adenosyl homocysteine (SAH). SAM is assessed by coupled enzyme reactions where SAH is hydrolyzed into adenosine and homocysteine by SAH hydrolyase, and homocysteine is cycled into the homocysteine conversion reaction to form a reaction cycle that amplifies the detection signal. The formed adenosine is immediately hydrolyzed into inosine and ammonia (NH₃). The enzyme glutamate dehydrogenase (GLDH) catalyzes the reaction of ammonia with 2-oxoglutarate and NAD⁺ to form NADH. Photometric measurement: the concentration of Hcy in the sample is directly proportional to the amount of NADH converted to NAD⁺.

Figure 6: Relative performance of the three analytical methods for measuring blood total homocysteine.

Abbreviations: HPLC, high-performance liquid chromatography.
CVD is the biggest killer in terms of global disease and its impact is predicted to grow due to the ageing populations of many countries. Commonly evaluated risk factors do not account for all cases of CVD.

Blood total homocysteine is a strong, independent risk factor for CVD. The relationship between elevated homocysteine and CVD is causal and probably due to multiple, potentially synergistic, pathogenetic mechanisms. Modest reduction in blood total homocysteine is predicted to confer large reductions in risk from CVD.

5 - <15 μmol/L is considered a normal fasting level of blood total homocysteine, although European laboratories tend to use a value of 12 μmol/L as the upper reference limit in adults. Upper reference limits depend on age and whether an individual has access to food fortified with folate or dietary supplements.

Measurement of blood total homocysteine is recommended for risk assessment in CVD patients, diagnosis of the rare genetic disease homocystinuria, and identification of individuals with (or at risk from) folate or cobalamin deficiency.

Homocysteine measurements may be useful to assist prevention and/or monitoring in many other clinical settings including: psychiatric illness, cognitive impairment in the elderly and Alzheimer’s disease, pregnancy complications, and diabetes mellitus.

The Roche Homocysteine enzymatic assay is a cutting-edge diagnostic tool for measuring blood total homocysteine concentrations in serum or plasma samples which offers:

- Excellent performance
- Precision over the entire measuring range
- Roche Homocysteine is more specific than other methods because it does not interfere by cystathionine
- Reliable results with optimized reproducibility enabling clinical decision in follow-up
- High efficiency
- All requested tests can be done out of one tube on a consolidated platform
- Consolidation of more than 130 clinical chemistry markers improves turnaround time
- Maximum convenience
- Cost, labour and time savings through optimized workflow
- Long on-board stability for cost-effective reagent usage

Summary