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

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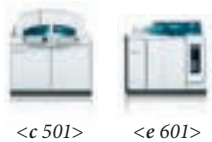
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cobas 8000 modular analyzer series
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>100 configurations

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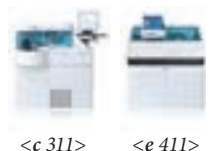

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cobas 4000 analyzer series
Low volume

3 configurations

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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 17.3 million deaths in 2008, which represents 30 % of the global total.[1] Of these deaths, an estimated 7.2 million were due to ischaemic heart disease (IHD) and 5.7 million were due to cerebrovascular disease (stroke).[2]
  - 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 $ 444.2 billion, which takes into account the cost of health services, medication, and lost productivity[3]

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.[4] 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.[5] 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.[6–10]

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[13–19]
- Deposition of cholesterol and other fats following degradation of dense aggregates formed from homocysteine thiolactone and low-density lipoprotein[10,20]
- Connective tissue changes induced by exposure and proliferation of the underlying smooth muscle and extracellular matrix[7,9]
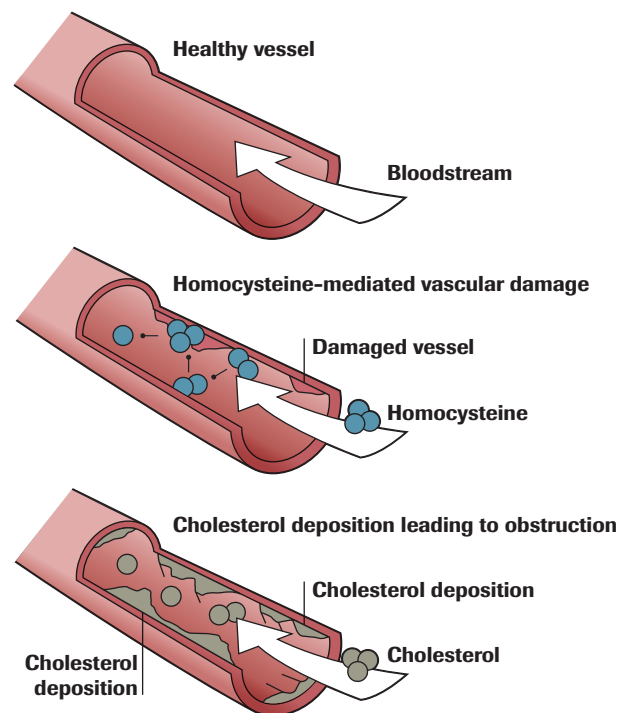

Figure 1: Excess circulating homocysteine increases the risk of cardiovascular disease

"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".
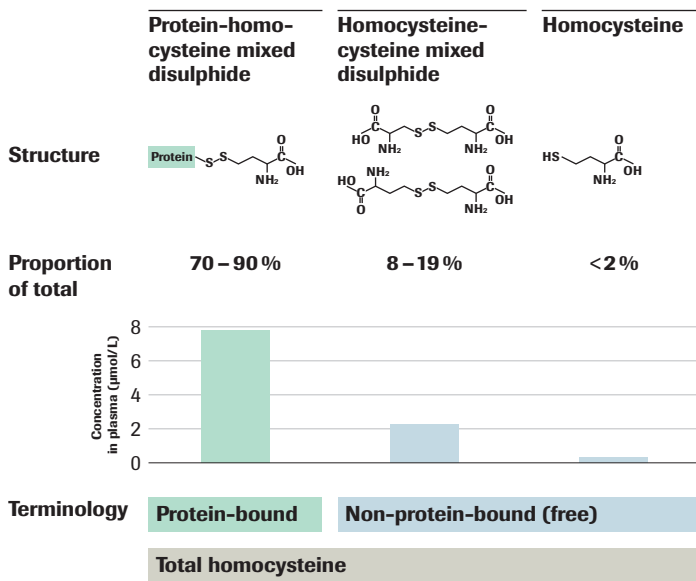

Figure 2: The three forms of homocysteine present within the circulation

Normal fasting levels of blood total homocysteine are considered to be <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.[9] 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).

Demographic group	Upper reference limit for total homocysteine (µmol/L)	
	Folate supplemented	Unsupplemented
Pregnant women	8	10
Children (<15 years)	8	10
Adults (15 – 65 years)	12	15
Elderly (>65 years)	16	20
Post–methionine load (4 – 6 hours)*	5-fold fasting level, or 40 µmol/L increase	

Table 1: Upper reference limits for blood total homocysteine [9]

*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.

Homocysteine in risk assessment and diagnosis

The American Association for Clinical Chemistry recommends measurement of blood total homocysteine in three clinical settings:[9]

- **Assessment as a risk factor for CVD**
- **Diagnosis of homocystinuria**
- **Identification of individuals with (or at risk of developing) folate (vitamin B9) or cobalamin (vitamin B12) 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).[9]

Category	Homocysteine level	Prevalence
Moderate	15 – 30 µmol/L	within the general population: <10 %
Intermediate	30 – 100 µmol/L	<1 %
Severe	>100 µmol/L	<0.02 %

Table 2: Classification of hyperhomocysteinemia

Circulating levels of total homocysteine can be influenced by, and be indicative of, a wide range of underlying physiologic and pathologic factors (Table 4 and Figure 3).

Target group	Rationale for testing	Frequency of testing
CVD patients or patients at high risk of CVD	Exclude homocystinuria and identify patients at high risk of CVD events and mortality	At entry into medical system, possibly every 3 – 5 years thereafter
Patients with symptoms of homocystinuria or a sibling with homocystinuria	Exclude or confirm homocystinuria	At entry into medical system
Patients with homocystinuria	Monitor treatment response and compliance	Every 2 – 4 weeks until values are stable, then annually or following change in treatment regimen
Patients with symptoms of, or at risk of, folate or cobalamin deficiency	Exclude or confirm deficiency	At entry into medical system; every 3 – 5 years in high-risk groups
Patients treated for folate or cobalamin deficiency	Monitor treatment response or detect relapse	2 – 4 weeks after initiation of therapy, then annually or when symptoms arise

Table 3: American Association of Clinical Chemistry recommendations for homocysteine testing [9]

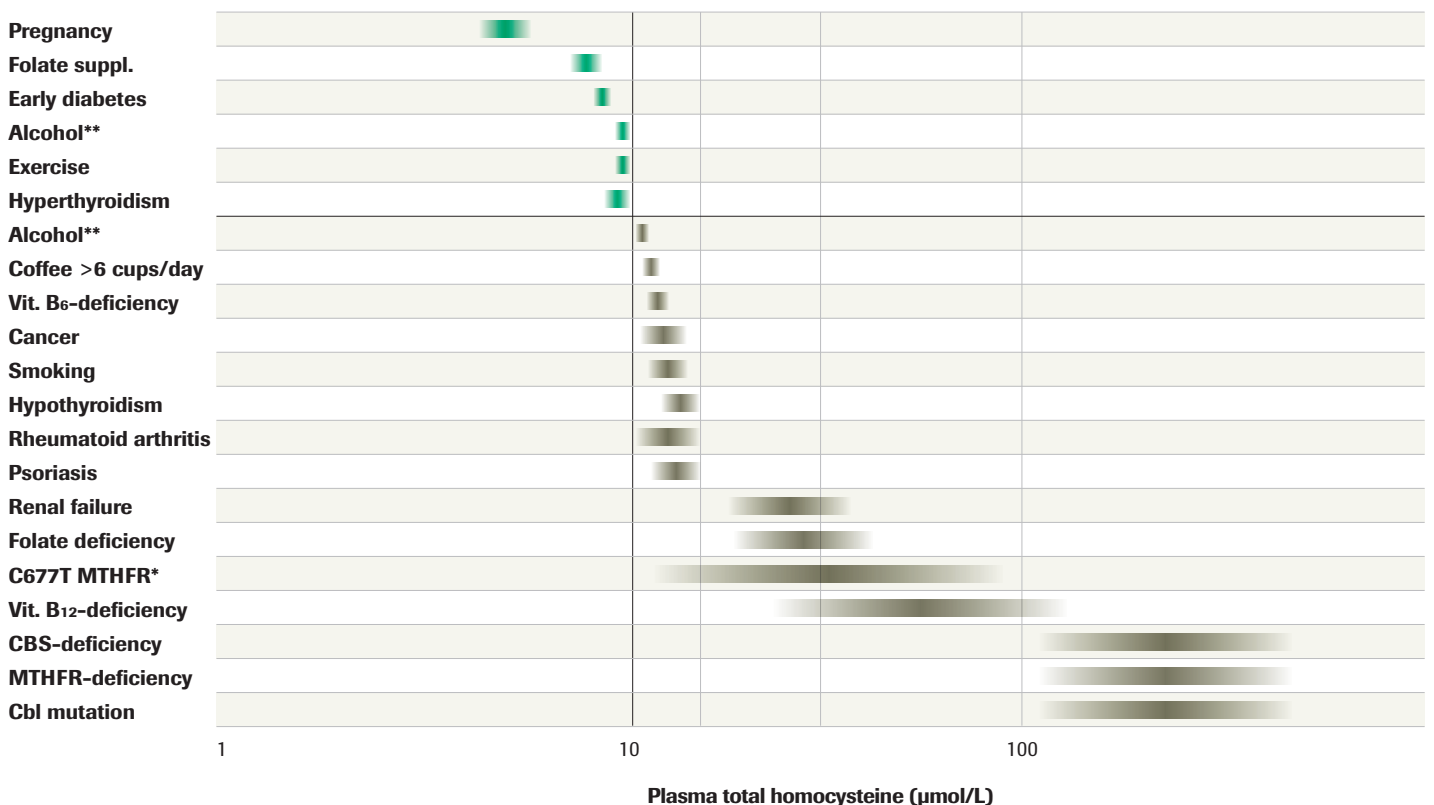Abbreviations: CVD, cardiovascular disease.

Pregnancy
Folate suppl.
Early diabetes
Alcohol**
Exercise
Hyperthyroidism
Alcohol**
Coffee >6 cups/day
Vit. B6-deficiency
Cancer
Smoking
Hypothyroidism
Rheumatoid arthritis
Psoriasis
Renal failure
Folate deficiency
C677T MTHFR*
Vit. B12-deficiency
CBS-deficiency
MTHFR-deficiency
Cbl mutation

1   10   100

Plasma total homocysteine (µmol/L)

Figure 3: Ranges of values for blood total homocysteine observed in individuals affected by different physiologic and pathologic factors [21]
*   combined with folate deficiency
** Effect of alcohol depends on whether consumption is high and chronic (increased total homocysteine) or moderate (decreased total homocysteine)

Abbreviations: CBS, cystathionine-β-synthase; MTHFR, N^5,N^{10}-methylenetetrahydrofolate reductase.

Patient factors	Effect upon blood total homocysteine levels				
	Decrease	Increase within reference range	Moderate hyperhomocysteinemia	Intermediate hyperhomocysteinemia	Severe hyperhomocysteinemia
Genetic					
CBS defects – homozygous					●
CBS defects – heterozygous*			●		
MTHFR defects – homozygous					●
MTHFR defects – heterozygous			●		
MTHFR – thermolabile mutation			●		
Cobalamin mutations					●
Down syndrome	●				
Physiologic					
Increasing age		●			
Male sex		●			
Impaired renal function		●			
Increasing muscle mass		●			
Pregnancy	●				
Lifestyle					
Deficient vitamin intake	●				
Smoking		●			
High caffeine consumption		●			
Alcohol consumption**	●		●		
Physical exercise	●				
Clinical					
Vitamin B_6 deficiency*			●		
Vitamin B_9 deficiency				●	
Vitamin B_{12} deficiency					●
Renal failure				●	
Hyperproliferative disorders			●		
Hypothyroidism			●		
Hyperthyroidism	●				
Early diabetes	●				
Drugs					
Folate antagonists (methotrexate)			●		
Vitamin B_6 antagonists*			●		
Vitamin B_{12} antagonists				●	
Adohomocysteine hydrolase inhibitors	●				
Antiepileptics (phenytoin)			●		
Contraceptives, hormone therapy	●				
Aminothiols	●				
Others (L-dopa, cholestyramine, niacin)			●		

Table 4: Patient factors influencing the level of blood total homocysteine [9]

Moderate, intermediate, and severe hyperhomocysteinemia defined by the ranges 15 – 30 µmol/L, 30 – 100 µmol/L, and >100 µmol/L, respectively.

* Individuals heterozygous for CBS defects or with vitamin B_6 deficiencies usually display normal fasting total homocysteine levels, but display an increased level following methionine load test.

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

Abbreviations: CBS, cystathionine-β-synthase; MTHFR, N^5, N^{10}-methylenetetrahydrofolate reductase.

Homocysteine in CVD risk reduction

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.[22] 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).
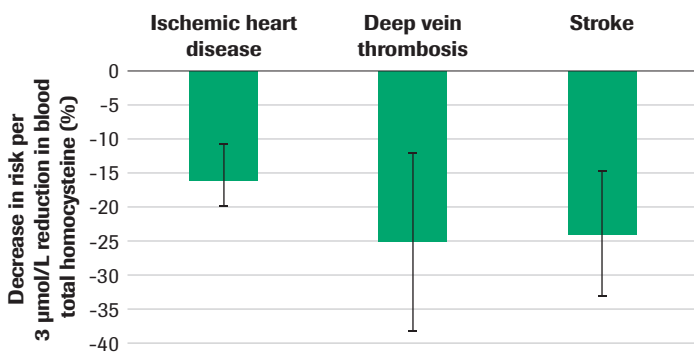
Figure 4: Predicted decrease in CVD risk following reduction in blood total homocysteine concentration[22]
Whiskers represent the upper and lower 95 % confidence intervals for the calculated decrease in risk.

"Modest reduction of homocysteine is predicted to reduce the risk up to 25 % for cardiovascular disease."[22]

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.[23] 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).
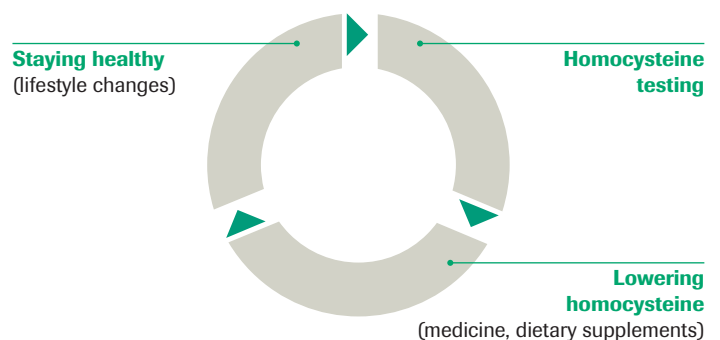

Figure 5: Lowering blood total homocysteine requires concerted efforts between clinicians and patients

Homocysteine in non-CVD settings

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

Homocystinuria[9]
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. Independent of the specific enzyme defect, these patients have a 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[9]
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 regions where a food fortification program has been introduced. For example, the prevalence in adults in the USA is currently <2%, whereas it was approximately 20% before fortification.

Cobalamin deficiency is most often observed in the elderly (prevalence: 10 – 15%), where it is nearly always attributable to malabsorption caused by gastric atrophy, ileal disease, or lack of intrinsic factor (pernicious anemia). Newborns also frequently exhibit low cobalamin. Age-independent causes of cobalamin deficiency include inadequate intake (e.g. vegetarians) or the use of certain drugs.

Renal failure[9]
There is an inverse relationship between renal function and blood total homocysteine; most dialysis patients (>85%) display hyperhomocysteinemia. Elevated blood total homocysteine is associated with an increased risk of hemodialysis access thrombosis, which is a common complication in dialysis patients.

Psychiatric disorders[9]
Elevated blood total homocysteine is associated with depression, especially in the elderly, as well as with schizophrenia. There is an inverse relationship in the elderly between cognitive scores and blood total homocysteine concentrations, as well as a dose-dependent association with Alzheimer's disease. Patients with vascular dementia or white matter disease also frequently exhibit high total homocysteine concentrations.

Pregnancy complications and birth defects[9]
Pregnant women have lower blood total homocysteine than women who are not pregnant, with concentrations >10 µmol/L rarely observed. Elevated blood total homocysteine during pregnancy is associated with an increased risk of placental vasculopathy, which can lead to preeclampsia, recurrent early pregnancy loss, premature delivery, low birth weight, and placental abruption or infarction. Women who have experienced previous pregnancy complications or had a child with a birth defect should be tested, as should those with (or at risk of developing) folate / cobalamin deficiencies.

Diabetes mellitus
The elevated levels of blood total homocysteine observed in patients with diabetes are believed to relate to the degree of diabetic nephropathy. Homocysteine concentrations are a greater risk factor for death in patients with type 2 diabetes compared with patients without diabetes.[24] Furthermore, the estimated survival time of patients with type 2 diabetes and a blood total homocysteine concentration >14 µmol/L is significantly shorter than patients with diabetes and concentrations <14 µmol/L.[25]

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* <P> 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.[9]

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.
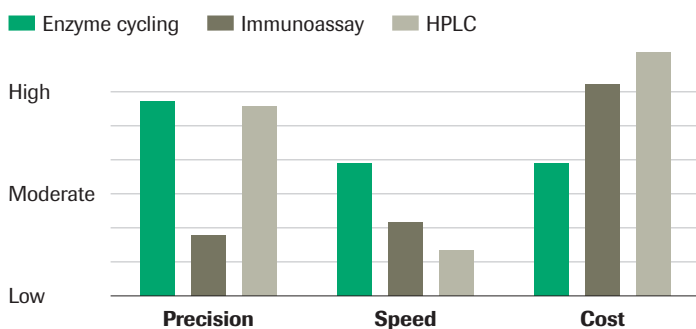

Figure 6: Relative performance of the three analytical methods for measuring blood total homocysteine [26]

*Abbreviations: HPLC, high-performance liquid chromatography.*

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).[27] 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 %.[28]

## Chemical principle of the enzyme cycling method[29]

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. **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), catalyzed by a Hcy S-methyl transferase (HMTase). SAH is assessed by coupled enzyme reactions where SAH is hydrolyzed into adenosine and homocysteine by SAH hydrolase, 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 (NH_3). The enzyme glutamate dehydrogenase (GLDH) catalyzes the reaction of ammonia with 2-oxoglutarate and NADH to form NAD_+. **Photometric measurement:** the concentration of Hcy in the sample is directly proportional to the amount of NADH converted to NAD_+ (ΔA_{340nm}).
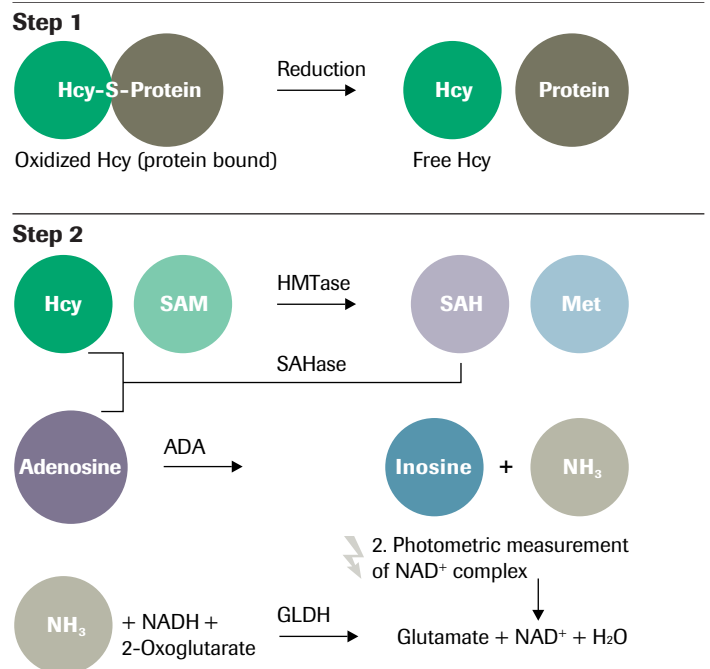
Summary

- 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
- <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[30]
    - 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
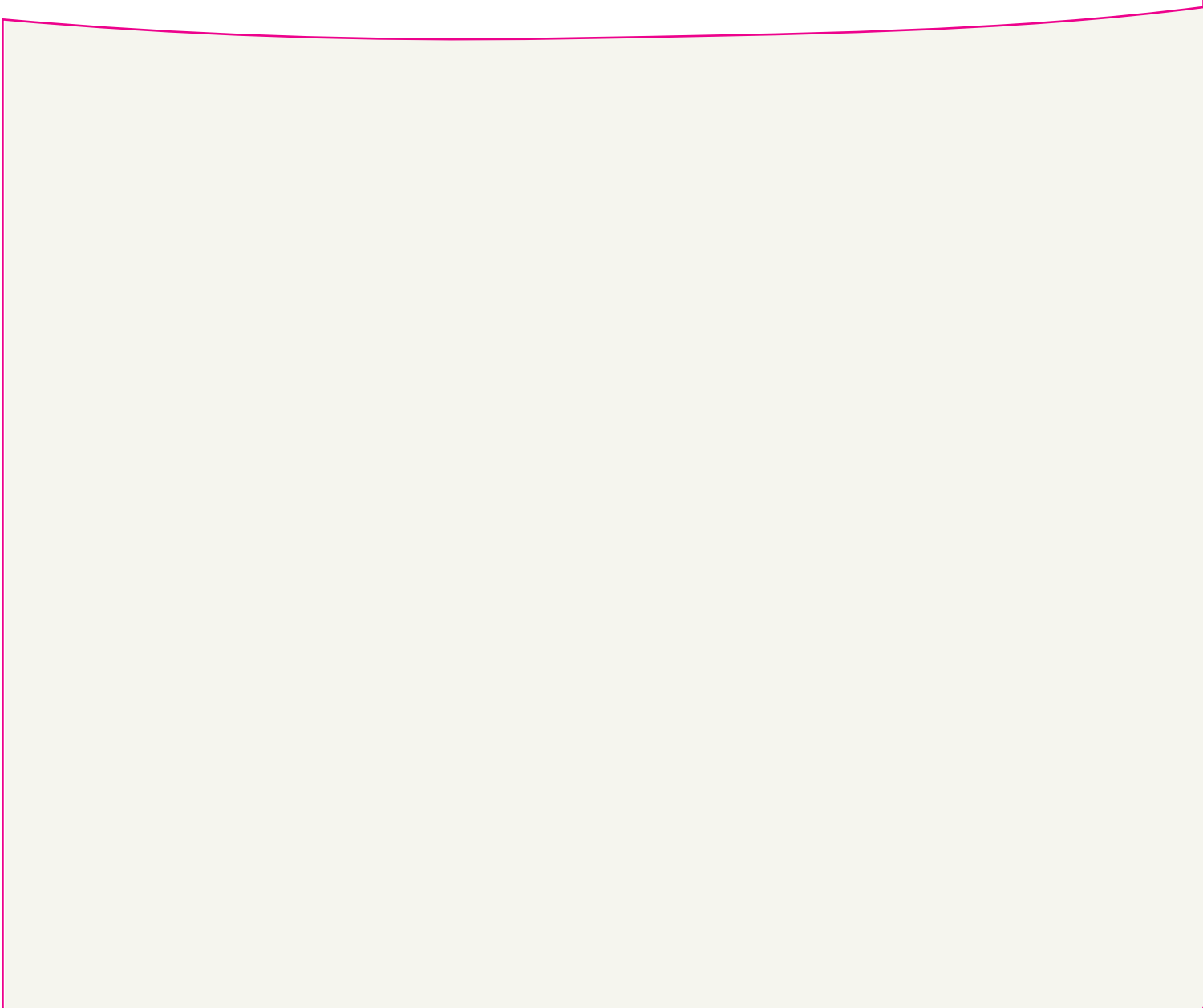
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